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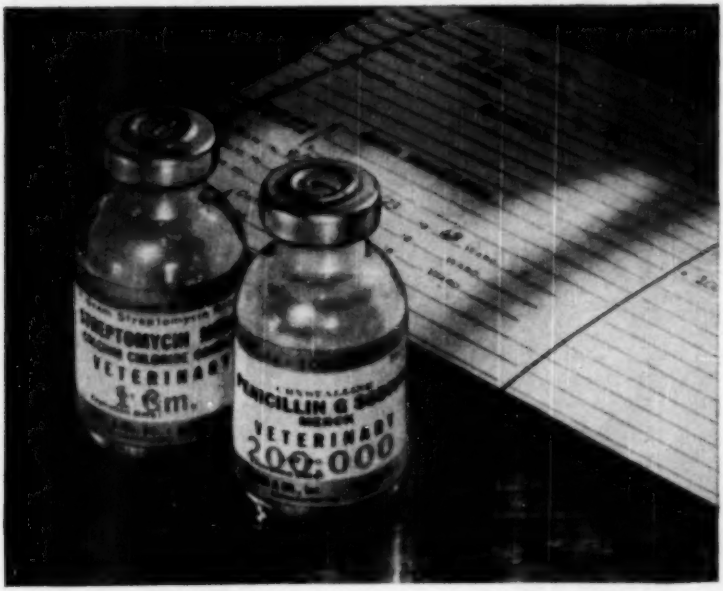

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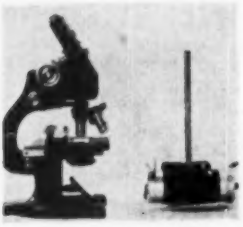

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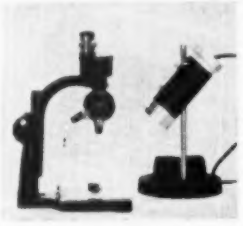

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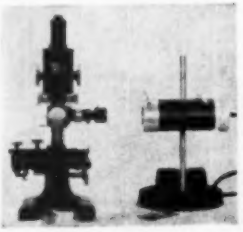

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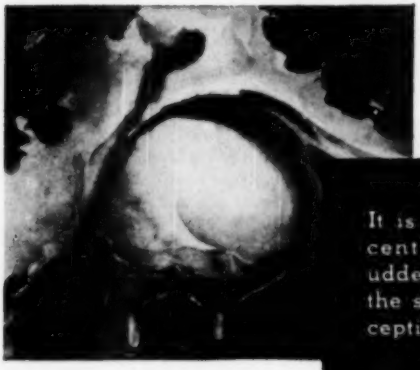

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The Transmission of Anaplasmosis

G. DIKMANS, D.V.M., Ph.D.
Beltsville, Maryland

In the fight against any infectious diseases, it is essential to know all possible sources of infection and all the means by which infection is disseminated.—P. J. DU TOIT (1923).

THE TRANSMISSION of anaplasmosis in nature remains an interesting and most puzzling problem. DuToit[8] stated that "In nature anaplasmosis is transmitted exclusively by ticks," but Sergent and coworkers,[42] after reporting various attempts to transmit this disease experimentally by ticks indigenous to North Africa and by stable flies, *Stomoxys calcitrans*, stated *la question du mode de transmission de l'anaplasmose dans la nature reste obscure et demande de nouvelles recherches*, that is, the question of the mode of transmission of anaplasmosis in nature remains unsolved and requires further research.

Anaplasmosis is a disease of worldwide occurrence and it has occupied the attention of investigators in many countries. The records of the work of these investigators, published in many languages, are found scattered throughout the world literature on this subject. Some of these records are readily available, others are relatively inaccessible. The purpose of this paper is to present the records of experiments dealing with the transmission of anaplasmosis and to analyze the results of some of these experiments, in order to facilitate the further research on this problem which is necessary.

One of the most extraordinary features of anaplasmosis is the readiness with which it has been transmitted mechanically by various types of surgical instruments and mechanically and biologically by various types of arthropods. This is a striking contrast to the methods of transmittal of such diseases as malaria, yellow fever, piroplasmosis, and theileriasis, which are transmitted under natural conditions by rather definite and circumscribed groups of insects and arachnids. Mosquitoes of the genera Anopheles and Aedes are practically the sole natural vectors of human malaria and yellow fever; ticks of the genus Boöphilus serve the world over as the natural vectors of bovine piroplasmosis; and the members of the tick genera Rhipicephalus and Hyalomma are the principal vectors of theileriasis; but it has been demonstrated experimentally that anaplasmosis can be transmitted by ticks belonging to different genera and by various kinds of flies and mosquitoes.

1) *Transmission of Anaplasmosis by Ticks.*—In areas where both piroplasmosis and anaplasmosis occur in cattle in the presence of the common vectors of piroplasmosis, namely *Boöphilus annulatus, Boöphilus microplus, Boöphilus decoloratus,* and *Boöphilus calcaratus,* these ticks have been considered, both on epizoötiologic grounds and on the basis of successful transmission experiments, as efficient vectors of anaplasmosis. However, in some experiments, it has not been easy to effect transmission by these ticks, and other genera and species of ticks have been shown experimentally to be potential natural vectors of this infection.

Results obtained by various investigators, in their attempts to transmit anaplasmosis by means of ticks, are shown in tables 1 and 1a.

In the experiments recorded in table 1a, only single stages of ticks were involved and the column "Stages of ticks involved in experiments" has therefore been omitted.*

The names of the ticks used in the tables and throughout the paper are the names used by the authors cited. The writer assumes no responsibility for their correctness.

From the Zoölogical Division, Bureau of Animal Industry, U. S. Department of Agriculture, Beltsville, Md.

*All experiments giving negative results, but in which the test animal was found to be immune or in which the susceptibility of the test animal was not ascertained, have been omitted from these tables.

TABLE 1—Results Obtained by Various Investigators with Ticks Tested Experimentally as Vectors of Anaplasmosis

Name of tick	Stages of ticks involved in experiments: Larva	Nymph	Adult	Egg	Larva	Nymph	Adult	No. of experiments	Result: Pos.	Result: Neg.	Author(s)	Year	Remarks
Boophilus decoloratus			A				B	3	3		Theiler, A.[42]	1912	
Boöphilus microplus			A				B	1	1		Quevedo, J. M.[39]	1916	Reported on authority of Brumpt, E., 1931.
B. microplus			A				B	1	1		Quevedo, J. M.[39]		Reported on authority of Brumpt, E., 1931.
B. microplus			A				B	3	3		Rosenbusch, F., and Gonzalez, R.[43]	1927	
B. microplus			A				B	1	1		Brumpt, E.[5]	1931	
Boöphilus calcaratus			A				B	2	2		Sergent, E. *et al.*[42]	1945	
Boöphilus annulatus			A				B	1	1		Rees, C. W.[39]	1934	
Hyalomma mauritanicum	A		A				B	2		2	Sergent, E. *et al.*[42]	1945	
H. mauritanicum			A				B	1		1	Sergent, E. *et al.*[42]	1945	
H. mauritanicum			A				B	1		1	Sergent, E. *et al.*[42]	1945	Larvae and nymphs engorging on susceptible animal did not transmit infection.
Hyalomma lusitanicum			A				B	1	1		Sergent, E. *et al.*[42]	1945	Larvae and nymphs engorging on susceptible animal did not transmit infection.
Rhipicephalus simus			A				B	1	1		Theiler, A.[42]	1912	
Rhipicephalus bursa	A		A				B	1	1		Brumpt, E.[5]	1921	
R. bursa	A		A				B	2		2	Sergent, E. *et al.*[42]	1945	
R. bursa			A				B	2		2	Sergent, E. *et al.*[42]	1945	
Rhipicephalus sanguineus	A		B					1	1		Rees, C. W.[35, 39]	1930	
R. sanguineus	A		B					4	2	2	Rees, C. W.[39]	1934	
R. sanguineus		A	B					2	2		Rees, C. W.[39]	1934	
R. sanguineus			A				B	2		2	Rees, C. W.[39]	1934	
R. sanguineus			A				B	1		1	Rees, C. W., and Avery, J. L.[38]	1939	
Dermacentor andersoni	A		B					1	1		Rees, C. W.[39]	1933	
D. andersoni	A		B					1	1		Rees, C. W.[39]	1934	
D. andersoni		A	B					2	2		Rees, C. W.[39]	1933	
D. andersoni		A	B					1	1		Boynton, W. H. *et al.*[3]	1938	
D. andersoni			A				B	1	1		Howell, D. E. *et al.*[33, 34]	1941	
D. andersoni			A				B	1		1	Rees, C. W., and Avery, J. L.[38]	1939	Larvae and nymphs also engorging on susceptible animals failed to transmit infection.
D. andersoni	A		B					1		1	Rozeboom, L. E. *et al.*[32]	1940	Larvae were progeny of adults that engorged on carrier animals.
D. andersoni		A	B					1		1	Rozeboom, L. E. *et al.*[32]	1940	Nymphs were progeny of adults that engorged on carrier animals. Larvae engorged on rabbits.

A = stage of tick engorging on infected animal; B = stage of tick engorging on susceptible animal.

TABLE I (continued)—Results Obtained by Various Investigators with Ticks Tested Experimentally as Vectors of Anaplasmosis

Name of tick	Stages of ticks involved in experiments: Larva	Nymph	Adult	Egg	Larva	Nymph	Adult	No. of experiments	Result: Pos.	Result: Neg.	Author(s)	Year	Remarks
D. andersoni			A----------B					1		1	Rozeboom, L. E.[22]	1949	Adults were progeny of females that had engorged on carrier animal. Larvae and nymphs engorged on rabbits.
Dermacentor variabilis	A---B							[illegible]	[illegible]		Rees, C. W.[21]	1932	
D. variabilis		A---B						[illegible]	[illegible]		Rees, C. W.[21]	1932	
D. variabilis		A---B						[illegible]	1		Rees, C. W.[20]	1934	
D. variabilis		A---B						[illegible]	[illegible]		Sanders, D. A.[26]	1933	
D. variabilis			A------B					[illegible]		2	Rees, C. W.[20]	1934	
D. variabilis			A------B---B---B					1		1	Rees, C. W., and Avery, J. L.[30]	1939	Ticks tested were progeny of adults that had engorged on carrier. Larvae and nymphs engorged on different susceptible animals, failed to transmit infection.
D. variabilis								1		1	Schmidt, H., and Piercy, P. L.[29]	1937	Transmission attempted from carrier animals.
D. variabilis										X	Piercy, P. L.[23]	1938	Number and details of experiments not given.
Dermacentor occidentalis			A------B					1	1		Boynton, W. H. *et al.*[3]	1936	
Dermacentor albipictus			A---B					1	1		Boynton, W. H. *et al.*[3]	1936	
Dermacentor nitens			A------B					1		1	Rees, C. W., and Avery, J. L.[30]	1939	
Ixodes ricinus			A------B					1	1		Zeller, H., and Helm, R.[37]	1923	
I. ricinus			A------B					1	1		Helm, R.[10]	1924	
I. ricinus										X	Piercy, P. L.[23]	1938	Number and details of experiments not given.
Ixodes scapularis	A---B							[illegible]	1	[illegible]	Rees, C. W.[20]	1934	
Ixodes sculptus		A---B						1		1	Rees, C. W.[20]	1934	
Amblyomma americanum	A---B							1		1	Rees, C. W.[20]	1934	
A. americanum		A---B						1		1	Rees, C. W.[20]	1934	
A. americanum			A------B					1		1	Rees, C. W.[20]	1934	
A. americanum	A---B									X	Piercy, P. L., and Schmidt, H.[28]	1941	Number of experiments not given.
Amblyomma maculatum	A---B							[illegible]		3	Rees, C. W.[20]	1934	
A. maculatum			A------B					1		1	Rees, C. W.[20]	1934	
A. maculatum										X	Piercy, P. L.[23]	1938	Number and details of experiments not given.
A. maculatum										X	Piercy, P. L., and Schmidt, H.[28]	1941	Number and details of experiments not given.
Amblyomma cajennense	A---B							[illegible]		3	Rees, C. W.[20]	1934	
A. cajennense			A------B					[illegible]		2	Rees, C. W.[20]	1934	
Ornithodoros megnini	A---B							[illegible]		1	Howell, D. E. *et al.*[11]	1942	
O. megnini			A----------B					1		1	Howell, D. E. *et al.*[11]	1942	

A = stage of tick engorging on infected animal; B = stage of tick engorging on susceptible animal.

The data presented in table 1 show that, under experimental conditions, anaplasmosis has been transmitted from infected to susceptible animals by various species of ticks. The successful transmission by *B. decoloratus*, *B. microplus*, *Rhipicephalus simus*, *Rhipicephalus bursa*, *Hyalomma lusitanicum* and *Ixodes ricinus*, and the negative results obtained with *B. calcaratus*, *R. bursa* and *Hyalomma mauritanicum*, have been listed on the authority of the authors whose names appear in the table.

With the possible exception of *I. ricinus*, the above mentioned ticks do not occur in the United States, and the present writer, not being familiar with conditions prevailing in the countries in which these ticks occur, does not feel competent to discuss their role as vectors under the natural conditions of their normal habitat.

Since the tick eradication campaign is practically completed, *B. annulatus* and *B. microplus* need no longer be considered as possible vectors of anaplasmosis in the United States in the greater part of the area originally included in the tick quarantine line. These ticks must, however, be considered as potential vectors in the limited areas in the United States in which they still occur and in borderline areas

TABLE 1a—Results Obtained by Various Investigators with Ticks Tested Experimentally as Vectors of Anaplasmosis

Name of tick	No. of experiments	Result		Author(s)	Year	Remarks
		Pos.	Neg.			
Dermacentor andersoni	1	1		Sanborn, C. E. *et al.*[28]	1938	Three partially engorged female ticks and 4 males removed from clinical case of anaplasmosis and placed, 6 days later, on susceptible animal. This animal developed anaplasmosis one month later.
D. andersoni	1	1		Sanborn, C.E. *et al.*[28]	1938	Male, used in previous experiment, removed from clinical case and placed on susceptible animal.
D. andersoni	1	1		Sanborn, C.E. *et al.*[28]	1938	Males used in two previous experiments placed on third susceptible animal.
D. andersoni	1	1		Rozeboom, L. E. *et al.*[22]	1940	Male removed from clinical case and placed, 2 hours later, on susceptible animal.
D. andersoni	1	1		Rozeboom, L. E. *et al.*[22]	1940	One male transferred from clinical case and placed, 1 day later, on susceptible animal.
D. andersoni	1	1		Rozeboom, L. E. *et al.*[22]	1940	Forty-one days elapsed between removal of tick from clinical case and its placement on susceptible animal.
Boöphilus calcaratus	1	1		Sergent, E. *et al.*[42]	1945	Immediate transfer of nymphs and adults from natural carrier to susceptible animal.
Rhipicephalus bursa	2	2		Sergent, E. *et al.*[42]	1945	Immediate transfer of adults from infected to susceptible animals.
R. bursa	2	2		Sergent, E. *et al.*[42]	1945	Immediate transfer of males from infected to susceptible animals.
Argas persicus	3	1	2	Howell, D. E. *et al.*[15]	1945	1) Ticks fed on infected animal for 2 days, kept in laboratory for 15 days, and then permitted to feed on susceptible animal for 2 days. Anaplasmosis transmitted. 2) Ticks fed on infected animal for from 1 to 5 minutes and then immediately transferred to susceptible animal. Time of feeding on susceptible animal not stated. Anaplasmosis *not* transmitted. 3) Ticks fed to repletion on infected animal, kept in laboratory for 32 days, and then permitted to feed on susceptible animal. Time of feeding on susceptible animal not stated. Anaplasmosis *not* transmitted.

subject to reinfestation. It is interesting to note that, of the eight attempts to transmit this infection experimentally with *B. annulatus*, only one was successful.

The table shows successful larva-to-nymph and nymph-to-adult transmission for *Rhipicephalus sanguineus*. No transovarian transmission could be demonstrated in three experiments. Successful larva-to-nymph, nymph-to-adult, and adult-to-larva (through the egg) transmissions are shown for *Dermacentor andersoni*, but failure to effect stage-to-stage and transovarian transmissions are also recorded. The record for *Dermacentor variabilis* shows successful larva-to-nymph and nymph-to-adult transmissions, but all attempts to show adult-to-larva transmission with this tick have failed. Failure to transmit the infection from carriers with this tick is also recorded. In the case of *Dermacentor occidentalis*, one successful adult-to-larva transmission is reported; also, in the case of *Dermacentor albipictus*, one nymph-to-adult transmission. Three larva-to-nymph attempts were made for *Ixodes scapularis;* one was successful, two were failures.

Since the ticks *D. occidentalis, D. andersoni, D. variabilis, D. albipictus, R. sanguineus*, and *I. scapularis*, in which either successful stage-to-stage or transovarian transmission has been demonstrated experimentally, occur in the United States, it appears to be in order to consider their possible role as vectors of anaplasmosis under natural conditions, on the basis of available evidence.

Dermacentor occidentalis must be considered as a potential vector within the range of its geographic distribution, because transmission of anaplasmosis from adult-to-larva has been demonstrated in this tick, and it has also been reported that all

TABLE 2—Instances of Transmission of Anaplasmosis by Unclean Surgical Instruments, Reported by Various Investigators

Means of transmission	No. of experiments	Result Pos.	Result Neg.	Author(s)	Year	Remarks
Goad used to drive oxen.				Descazeaux, J.[9]	1924	
Instruments used in dehorning.				Sanborn, C. E.[30]	1930	Transmission unintentional.
Instruments used in dehorning.				Hilts, W. H.[31]	1928	Transmission unintentional.
Infected lancet.	2	2		Rees, C. W.[26, 28]	1930	Immediate transfer from infected to susceptible animal.
Needles and syringes used to vaccinate animals for foot-and-mouth disease.				Leclainche, E.[19]	1930	Vaccine became contaminated with blood. Transmission unintentional.
Needle.				Sergent, E. *et al*.[41]	1924	
Needle.	1	1		Julius, G. A.[16]	1934	Time elapsing between stabbing of infected and noninfected animals: 2 seconds. Needle introduced deeply enough to draw blood.
Needle.	1		1	Julius, G. A.[16]	1934	Needle not permitted to penetrate more than 2 mm.
Needle.	1		1	Julius, G. A.[16]	1934	Needle penetration as in first experiment, but time elapsing between stabbing of infected and noninfected animals, 10 seconds.
Instruments used in dehorning and horn tipping.				Stiles, G. W.[40]	1936	Transmission unintentional. Author reports 10 outbreaks involving 1,500 animals, 165 cases of anaplasmosis developed with 55 deaths.
Vaccination and intravenous injection procedures.				Stiles, G. W.[40]	1936	Transmission unintentional. Author reports 105 cases of anaplasmosis following vaccination and intravenous injection procedures.
Stabbing with pitchfork.				Stiles, G. W.[40]	1936	Transmission unintentional.
Instruments used in castration.				Stiles, G. W.[40]	1936	Transmission unintentional.
Instruments used in dehorning.				Goss, L. W. *et al*.[8]	1941	Transmission unintentional.

stages (larva, nymph, and adult) have been found on cattle.

The role of *D. andersoni* as a possible vector under natural conditions is in need of further investigation. There appears to be some correlation between the seasonal occurrence of the adults of *D. andersoni* on cattle and the seasonal occurrence of anaplasmosis in some areas of the geographic range of this tick. Stage-to-stage and adult-to-larva transmissions have been demonstrated experimentally. However, the larvae and nymphs have not yet been reported as occurring on cattle and, according to Cooley,[4] it takes two years to complete one generation. Unless, therefore, it can be shown that the larval and nymphal stages occur on cattle with some degree of regularity, or that the etiologic agent of anaplasmosis is passed from adult to adult, regardless of the hosts upon which the larvae and nymphs engorge, judgment on the role of this tick in the transmission of anaplasmosis under natural conditions must be reserved. Transmission of anaplasmosis by male *D. andersoni*, even when several weeks intervened between their removal from an infected host and their placement on a susceptible host, has also been demonstrated. It has been postulated that, under natural conditions, males may be brushed off one animal and reattached on another and that the disease may be transmitted in this manner. This is an intriguing possibility but there is, unfortunately, no information available as to how frequently such transfers from host to host occur under natural conditions.

Dermacentor variabilis has a much wider geographic distribution in the United States than either *D. occidentalis* or *D. andersoni*. Stage-to-stage transmission has been demonstrated for this tick, but adult-to-larva transmission has not been accomplished. The difficulties that prevent the acceptance of *D. andersoni* as a proved vector of anaplasmosis under natural conditions also prevent the acceptance of *D. variabilis* as a natural vector. These difficulties are that the larvae and nymphs do not, so far as is known, occur on cattle; and until it has been shown that transmission can take place from adult (through egg) to larva or from adult to adult, even though larvae and nymphs engorge on rodents, the specific role of this tick as a possible natural vector cannot be settled.

Dermacentor albipictus, the winter tick, is a one-host tick, widely distributed in the northern and western states and Canada. If *Dermacentor nigrolineatus* is considered to be identical with *D. albipictus*,[5] the distribution is extended to all parts of the United States.[1] Stage-to-stage, nymph-to-adult transmission has been demonstrated experimentally for this tick. In some parts of its geographic range, in which anaplasmosis is known to occur, its seasonal occurrence coincides with the seasonal occurrence of this disease.[12] In those areas, this tick must, therefore, be considered as a potential vector, but information available at the present time is inadequate to assess its importance as a vector under natural conditions.

It is impossible to determine the importance of *I. scapularis* as a possible vector of anaplasmosis on the basis of available information. However, this is a three-host tick with larvae and nymphs found on animals other than cattle, and the seasonal occurrence of the adults on cattle is not correlated with the seasonal occurrence of anaplasmosis. Further investigation is, therefore, necessary to determine whether or not this tick should be considered in the epizoötiology of this disease.

Rhipicephalus sanguineus, while present in areas where anaplasmosis is enzoötic, occurs on dogs. Its occurrence on cattle is extremely rare, and on the basis of present information, it does not need to be considered in the epizoötiology of anaplasmosis.

Experiments dealing with the transmission of anaplasmosis by single stages of ticks are listed in table 1a.

In the experiments of Sanborn and coworkers,[38] there was an interval of six days between the removal of the ticks from an animal dying of anaplasmosis in Wyoming and their placement on a susceptible animal in Oklahoma. The same males which participated with the females in transmitting anaplasmosis to the first experimental animal subsequently transmitted the disease to 2 other animals in succession.

In the experiments by Rozeboom and his coworkers,[32] the time intervals between the feedings of the male ticks on infected and susceptible animals were two hours, twenty-four hours, and forty-one days. Furthermore, in two of the three experiments, only 1 male was used in each. The time intervals between successful transfer feedings in the experiments reported by the above mentioned authors are in striking contrast to the time intervals recorded in table 4 in the successful transfer feedings with tabanids. In the experiments with tabanids, no transmission was obtained when more than five minutes elapsed between the feedings on infected and susceptible animals.

The experiments by Sanborn,[36] Rozeboom,[32] and their coworkers give point to the argument that within the geographic

range of *D. andersoni*, male ticks may be important vectors of anaplasmosis, if it is assumed that, under natural conditions, ticks readily transfer from animal to animal. The evidence in favor of this assumption is rather limited at this time, but Howell[12] reported finding nymphs and adults of *D. albipictus* in closely cropped pasture land. He also reported that under conditions of close association, such as obtain in feeding corrals, *D. albipictus* readily transferred from infested to clean animals. It should, however, be noted that Sergent and coworkers,[42] in commenting on their experimental transmission of anaplasmosis by male *R. bursa*, expressed the opinion that, in nature, such transmission is without doubt the exception rather than the rule.

Argas persicus, the fowl tick, is probably not an important vector of anaplasmosis, because it normally occurs on birds and not on cattle.

Sanborn and Moe[37] reported having tested the following ticks experimentally as vectors of anaplasmosis: *Amblyomma americanum, Amblyomma cajennense, D. albipictus, D. nigrolineatus, Dermacentor nitens, Dermacentor parumapertus, D. variabilis, Dermacentor venustus, Haemaphysalis leporis-palustris, I. ricinus, I. scapularis, Ixodes sculptus, Margaropus annulatus, Ornithodoros megnini, Ornithodoros turicata* and *R. sanguineus*. According to the report, anaplasmosis was not transmitted by any of these ticks. No details of experiments are given.

2) *Transmission by Infected or Unclean Instruments.*—In table 2 are recorded instances of transmission of anaplasmosis, both intentional and unintentional, by infected or unclean instruments used in surgical procedures.

Examination of the material presented in table 2 shows that mechanical transmission of anaplasmosis by unclean instruments is not only possible, but may occur readily.

3) *Transmission by Insects.*—In table 3* are presented the recorded attempts to transmit anaplasmosis by horseflies, Tabanidae, *Tabanus* spp., *Chrysops* spp., *Silvius pollinosus* and *Haematopota pluvialis;* stable flies, *S. calcitrans;* horn flies, *Siphona irritans* (*Haematobia irritans*); mosquitoes, *Psorophora columbiae, Psorophora ciliata, Psorophora signipennis, Psorophora cyanescens, Psorophora discolor* and *Aedes aegypti;* and lice, *Haematopinus* sp. and *Linognathus* sp.

The present writer assumes no responsibility for the correctness of the names of the insects listed in the table.

The material presented in table 3 indicates that experimental transmission by insects has, with few exceptions, been successful only when the insects, chiefly tabanids, were allowed to engorge partially on animals in the clinical stages of anaplasmosis and were then transferred, without undue delay, to susceptible animals to complete their engorgement. No transmission occurred when the interval between the feeding on the infected animal and the transfer to the susceptible animal was five minutes or more, nor when the susceptible animal was subjected to less than 13 bites from the flies that had fed on an infected animal.

In the table are listed five successful experimental transmissions from carrier animals.** In the 3 cases of successful transfer from carrier animals reported by Howell and coworkers,[13, 14] previously reported by Sanborn and coworkers,[33, 34, 35] the source animal for at least 2 of these cases, and probably for all 3, was 1 bull. This bull was born on Aug. 3, 1929, and used as a source animal on Aug. 17 and 19, 1930. He was reported to have been prenatally infected, his dam having been injected with virulent blood on July 22, 1929. According to the record, this bull was injected with virulent blood but showed no clinical response to this injection. The date of this injection is not recorded and there is no record of any examination of the blood to determine whether there was any parasitic response. The infection was transmitted from this animal on Aug. 19, 1930, to cow 21.[13] The next definitely established transmission from this animal occurred in July, 1931, and was reported in 1932.[35] In the third case,[14] previously reported by Sanborn and coworkers,[33, 34, 35, 36] it is probable that this bull also served as the source animal.

Howell *et al.*[13, 14] mention using several other carriers as source animals in their experiments, but they were apparently not successful in transmitting the disease from any carrier animal other than the above mentioned bull.

The other two successful experimental transmissions from a carrier animal are those reported by Lotze.[20] In these cases, successful transmission was accomplished when there was what has been termed by Sergent and coworkers, a *rechute parasitaire*, that is, a reappearance of parasites

*All experiments giving negative results, but in which the test animal was found to be immune or in which the susceptibility of the test animal was not ascertained, have been omitted from this table.

**A carrier animal is defined by Howell and coworkers[13, 14] as an animal that has recovered from an attack of anaplasmosis and no longer shows any definite microscopic or clinical evidence of the disease.

in the blood of the peripheral circulation in sufficient numbers that they can be detected without much effort by a microscopic examination of the blood. The significance of Lotze's findings consists in the fact that they suggest a possible explanation for the occurrence, recurrence, and spread of the disease under natural conditions. Sergent and coworkers[41] reported that 31 animals, in which the dates of the initial infections were known, showed Anaplasma in the blood of the peripheral circulation on an average of three days per month for nine months after recovery from the initial attack. Some animals, in which the dates of the initial, natural infection were not known, showed Anaplasma in the peripheral circulation during a period of observation of twelve months, the average number of days per month on which they were found varying from 9 in the first month to 0.7 in the twelfth month. In 2 animals, observed for a period of thirty-two months after the termination of the initial attack, Anaplasma organisms were found in the peripheral circulation, in 1 case for one day during the twenty-ninth month and in the other for one day during the twenty-sixth month of observation. These authors also reported (*loc. cit.*) the occurrence of *recrudescence printanière du parasitisme*, or springtime recurrence of parasites (Anaplasma) occurring from February to March in 7 of 18 cases (39%). These relapses lasted from five to twenty-one days and the number of Anaplasma organisms varied from 4 to 20 per 1,000 red blood cells. There is no information from American sources as to how frequently such relapses occur, but that they do occur is evident from Lotze's experiments.[19, 20]

There are many outbreaks of anaplasmosis for the beginning of which it is often difficult and sometimes impossible to find any logical explanation at the time of their occurrence. Usually, no ticks are present on any of the animals. It has been argued that the absence of ticks cannot be considered as evidence that ticks did not initiate the outbreak, because the length of the incubation period would permit the ticks to complete their engorgement and drop off before any clinical evidence of the disease makes its appearance. This argument may be valid in areas where ticks that have been demonstrated to be potential vectors occur. The outbreaks, however, are not confined to those areas. They are found in places where ticks do not occur on large domestic animals or where the ticks that do occur, cannot, on the basis of available evidence, be considered as proved vectors under natural conditions.

When ticks can be eliminated as possible or probable vectors, the possibility of mechanical transmission by unclean instruments must be considered, and, for such transmission to occur, the presence of an infected animal in the herd is necessary. It is usually not too difficult to determine by judicious inquiry whether such an animal is present in the herd and whether operations involving the possible transfer of blood from this infected animal to other animals in the herd have been performed thirty to sixty days previous to the outbreak.

When mechanical transmission by unclean instruments can be eliminated, the possibility of transmission by insects must be considered. For such transmission to take place, an infected animal must be available either in the herd in which the disease occurs or in a herd in close proximity. If this infected animal is in the clinical stages of the disease (and this could occur if it were part of a herd occupying pastures adjoining those used by the herd in which the disease is present), the explanation for the outbreak would not be difficult, because it has been clearly shown that flies, especially tabanids, can transmit the disease readily from clinical cases. The owner of the herd in which the disease is being investigated might be completely unaware of the existence of this clinical case because, if the herd did not belong to him, he would have no reason to be concerned about it. However, if the infected animal is a carrier, and this appears more often to be the case, it is more difficult to explain the transmission to susceptible animals, because of the meager success that has attended experimental efforts to transmit the disease from carriers by flies. Because of this difficulty, it has been argued that a clinical case, manmade, but probably mild in character and therefore unobserved, is necessary for the start of an outbreak. The outbreak, once started, could then be easily disseminated by the flies.

It seems, however, that the observations of Sergent and coworkers[41] and those of Lotze[20] offer an acceptable explanation for the beginning of these outbreaks. These authors reported the occurrence in carrier animals of relapses with parasites appearing in the blood of the peripheral circulation in sufficient numbers to be readily detectable. Lotze demonstrated that these parasites could be transmitted by tabanids, and it is not unreasonable to suppose that, in this manner, the disease may be built up from late spring through summer, to reach serious proportions in the late summer and fall.

TABLE 3—Results Obtained by Various Investigators with Insects Tested Experimentally as Mechanical Vectors of Anaplasmosis

Name of insect	No. of experiments	Result		Author(s)	Year	Remarks
		Pos.	Neg.			
Tabanus abactor *Tabanus equalis* *Tabanus erythraeus*	4 1 1		4 1 1	Howell, D. E. *et al.*[15, 16]	1941	Deferred feeding only from clinical cases to susceptible animals.
T. abactor	1		1	Howell, D. E. *et al.*[15, 16]	1941	Deferred feeding only from carrier to susceptible animal.
Tabanus sulcifrons	3		3	Lotze, J. C., and Yeagley, M. J.[19]	1941	Immediate transfer feeding by 16 to 24 flies from infected animal 2 and 3 days after Anaplasma had appeared in red blood cells.
T. sulcifrons	6	2	4	Lotze, J. C.[20]	1944	Immediate transfer feedings from carriers failed, except when Anaplasma reappeared and could be demonstrated in red blood cells during subclinical parasitic relapse.
Stomoxys calcitrans	?		X	Rosenbusch, F., and Gonzalez, R.[21]	1927	Authors use term "Mosca brava."
S. calcitrans	1	1		Sanders, D. A.[22]	1933	Immediate transfer feeding by several hundred flies from clinical case to susceptible animal. Anaplasma found in blood of susceptible animal 12 days after transfer feeding. No clinical symptoms noted.
S. calcitrans	8		8	Julius, G. A.[23]	1934	Freshly bred stable flies used in immediate and deferred transfer feedings. Numbers not stated.
S. calcitrans	1		1	Taylor, E. L.[24]	1935	Immediate transfer feedings from clinical case to susceptible animal during a period of 18 days.
Tabanus fumipennis	1	1		Sanders, D. A.[22]	1933	Immediate transfer feeding by about 100 flies. Disease developed 62 days after first exposure. Presence of Anaplasma demonstrated. No clinical symptoms observed.
Tabanus circumdatus	1		1	Julius, G. A.[23]	1934	
Tabanus atratus	4	1?	3	Morris, H. *et al.*[25]	1936	Immediate transfer feeding from clinical case of Anaplasmosis to susceptible animal, by about 15 flies in each experiment.
Tabanus fuscicostatus	3		3	Morris, H. *et al.*[25]	1936	Seventy-five flies used as in experiment with *T. atratus*.
T. atratus *Tabanus lineatus* *Chrysops nigra* *Chrysops* sp.	1		1	Schmidt, H., and Piercy, P. L.[26]	1937	Immediate transfer feeding from carrier to susceptible animals.
T. atratus	1		1	Schmidt, H., and Piercy, P. L.[26]		Immediate transfer feeding from carrier to susceptible animals.
T. sulcifrons	1		1	Schmidt, H., and Piercy, P. L.[26]		Immediate transfer feeding from carrier to susceptible animals.
Chrysops sp.	1		1	Schmidt, H., and Piercy, P. L.[26]		Immediate transfer feeding from carrier to susceptible animals.
Tabanus americanus	1	1		Howell, D. E. *et al.*[15, 16]	1941	Immediate transfer feeding from clinical cases to susceptible animals.
Tabanus oklahomensis	1	1		Howell, D. E. *et al.*[15, 16]		Immediate transfer feeding from clinical cases to susceptible animals.
T. sulcifrons	2	1	1	Howell, D. E. *et al.*[15, 16]		Immediate transfer feeding from clinical cases to susceptible animals.
T. abactor	4	2	2	Howell, D. E. *et al.*[15, 16]		Immediate transfer feeding from clinical cases to susceptible animals.
T. atratus	1		1	Howell, D. E. *et al.*[15, 16]		Immediate transfer feeding from clinical cases to susceptible animals.

TABLE 3 (continued)—Results Obtained by Various Investigators with Insects Tested Experimentally as Mechanical Vectors of Anaplasmosis

Name of insect	No. of experiments	Result Pos.	Result Neg.	Author(s)	Year	Remarks
T. sulcifrons	1	1		Howell, D. E. *et al.*[15, 16]	1941	Immediate transfer feeding from carriers to susceptible animals.
T. erythraeus	2		2	Howell, D. E. *et al.*[15, 16]		Immediate transfer feeding from carriers to susceptible animals.
Tabanus rubescens	1		1	Howell, D. E. *et al.*[15, 16]		Immediate transfer feeding from carriers to susceptible animals.
T. atratus	1		1	Howell, D. E. *et al.*[15, 16]		Immediate transfer feeding from carriers to susceptible animals.
Chrysops sp. *T. atratus* *T. erythraeus*	1		1	Howell, D. E. *et al.*[15, 16]		Immediate transfer feeding from carriers to susceptible animals.
T. abactor	1	1		Howell, D. E. *et al.*[15, 16]		Immediate and deferred transfer feeding from clinical to susceptible animals.
T. sulcifrons	2	2		Howell, D. E. *et al.*[15, 16]		
T. equalis	2	2		Howell, D. E. *et al.*[15, 16]		
T. erythraeus	1	1		Howell, D. E. *et al.*[15, 16]		
T. fuscicostatus	1		1	Howell, D. E. *et al.*[15, 16]		
T. abactor *T. sulcifrons* *T. fuscicostatus* *Tabanus venustus* *Silvius pollinosus*	1	1		Howell, D. E. *et al.*[15, 16]		Immediate and deferred transfer feeding from clinical to susceptible animals.
Chrysops sp.	1		1	Howell, D. E. *et al.*[15, 16]		Immediate and deferred transfer feeding from clinical to susceptible animals.
T. abactor *T. sulcifrons* *Chrysops sequax*	1	1		Howell, D. E. *et al.*[15, 16]		Immediate and deferred transfer feeding from clinical to susceptible animals.
T. lincola *T. sulcifrons* *T. atratus*	1		1	Howell, D. E. *et al.*[15, 16]		Immediate and deferred transfer feeding from carriers to susceptible animals.
T. venustus	1	1		Howell, D. E. *et al.*[15, 16]		Immediate and deferred transfer feeding from carriers to susceptible animals.
Psorophora columbiae *Psorophora signipennis* *Psorophora cyaneacens* *Psorophora ciliata* *Psorophora discolor*	1		1	Howell, D. E. *et al.*[15, 16]	1941	Immediate transfer feeding from carrier to susceptible animal by 496 individuals of 5 species of Psorophora. Three interrupted feedings at 3- and 2-day intervals.
P. columbiae *P. ciliata* *Aedes aegypti*	1	1		Howell, D. E. *et al.*[15, 16]	1941	*P. columbiae* and *P. ciliata*, 198 individuals, permitted to engorge partially for 4 days and then immediately transferred to susceptible animals. *Aedes aegypti*, 21 individuals, used in similar manner for 3 days, and *P. ciliata*, 22 individuals for 1 day.
P. columbiae *P. ciliata*	1	1		Howell, D. E. *et al.*[15, 16]	1941	*P. columbiae* and *P. ciliata*, 1,525 individuals, permitted to engorge partially on clinical case for 3 continuous days and then, each day, immediately transferred to susceptible animal.
S. calcitrans	1		1	Sergent, E. *et al.*[42]	1945	Six immediate transfer feedings on 6 separate days from clinical case to susceptible animal. Number of flies used not stated.
Haematopota pluvialis	1		1	Taylor, E. L.[48]	1935	Forty-six immediate transfer feedings over a period of 20 days, from clinical case to susceptible animal.
Siphona irritans (*Haematobia irritans*)	3		3	Morris, H. *et al.*[29]	1926	Immediate transfer feeding from clinical case to susceptible animal. One hundred and fifty flies used over a period of 3 days.

TABLE 3 (continued)—Results Obtained by Various Investigators with Insects Tested Experimentally as Mechanical Vectors of Anaplasmosis

Name of insect	No. of experiments	Result Pos.	Result Neg.	Author(s)	Year	Remarks
Haematopinus sp.			X	Rosenbusch, F., and Gonzalez, R.[82]	1927	Authors report "experiments with lice were unsuccessful."
Linognathus vituli			1	Velasquez, Jose Q.[90]	1938	Results of transfer feeding from infected to susceptible animal negative, but author reports having produced the disease by subcutaneous injection of macerated lice, *Linognathus vituli*, that had fed on anaplasmosis-infected animal.

The insects experimentally determined as potential vectors of anaplasmosis act, *so far as known*, like needles, lancets, and other surgical instruments, only as mechanical transfer agents, with no conservation or developmental stages of the etiologic agent of the disease, whatever may be its nature, taking place within them. In this respect, they differ markedly from the insect vectors of malaria, filariasis, onchocerciasis, and trypanosomiasis, in which definite developmental stages of the causative organisms of these diseases occur. From the viewpoint of the conservation and maintenance of the etiologic agent of anaplasmosis it would, therefore, not be illogical to assume that, in nature, ticks act as vectors of this disease, especially in areas where the disease occurs enzoötically, since it has been demonstrated experimentally that in male ticks the etiologic agent can be conserved for relatively long periods, and that in female ticks it can pass transovarially from adult to larva and through the various developmental stages from larva to imago.

However, at the present time, we can only say, with Sergent and coworkers (*loc. cit.*), *la question du mode de transmission de l'anaplasmose dans la nature reste obscure et demande des nouvelles recherches.*

References

[1]Bishopp, F. C., and Trembley, H. L.: Distribution and Hosts of Certain American Ticks. J. Parasitol., *31*, (1945): 1-54.

[2]Boynton, W. H., Herms, W. B., Howell, D. E., and Woods, Gladys M.: Anaplasmosis Transmission by Three Species of Ticks in California. J.A.V.M.A., *88*, (1936): 500-502.

[3]Brumpt, E.: Transmission d'*Anaplasma Marginale* par *Rhipicephalus Bursa* et par Margaropus. Ann. de parasitol., *9*, (1931): 4-9.

[4]Cooley, R. A.: The Rocky Mountain Wood Tick, Montana State Coll. and Agric. Exp. Sta., Bull. *248*, (1932): 58.

[5]Cooley, R. A.: The Genera Dermacentor and Otocentor (Ixodidae) in the United States, with Studies in Variation. Nat. Inst. of Health, Bull. *171*, U. S. Pub. Health Serv., (1938): 89.

[6]Descazeaux, J.: L'Anaplasmose au Chili. Bull. Soc. path. exot., *17*, (1924): 639-642.

[7]DuToit, P. J.: Sheep Scab: The Infectivity of Kraals. Ninth and Tenth Reports, Director Vet. Education and Res., (1923): 223-229.

[8]DuToit, P. J.: Anaplasmosis. Twelfth Internat. Vet. Cong., *3*, (1935): 325-348.

[9]Ross, L. W., Mills, J. W., and Guard, W. F.: Anaplasmosis Recognized in Ohio. J.A.V.M.A., *98*, (1941): 151.

[10]Helm, R.: Beitrag zum Anaplasmen-Problem. Ztschr. f. Infektionskr., *25*, (1924): 199-226.

[11]Hilts, W. H.: Anaplasmosis Following Dehorning. Cornell Vet., *18*, (1928): 330-332.

[12]Howell, D. E.: The Ecology of *Dermacentor Albipictus* (Packard). Proc. Sixth Pacific Sci. Cong., *4*, (1940): 439-458.

[13]Howell, D. E., Stiles, G. W., and Moe, L. H.: The Transmission of Anaplasmosis by Mosquitoes (Culicidae). J.A.V.M.A., *99*, (1941): 107-110.

[14]Howell, D. E., Sanborn, C. E., Rozeboom, L. E., Stiles, G. W., and Moe, L. H.: The Transmission of Anaplasmosis by Horseflies (Tabanidae). Okla. A. and M. Coll. and Agric. Exper. Sta., Tech. Bull. T-11, (1941): 1-23.

[15]Howell, D. E., Stiles, G. W., and Moe, L. H.: The Fowl Tick, (*Argas Persicus*), a New Vector of Anaplasmosis. Am. J. Vet. Res., *4*, (1943): 73-75.

[16]Julius, G. A.: Eighth Ann. Rep. Council Scient. and Indust. Res., Commonwealth of Australia, (1924): 21-22.

[17]Julius, G. A.: Ninth Ann. Rep. Council Scient. and Indust. Res., Commonwealth of Australia, (1935): 28-34.

[18]Læclainche, E.: Un foyer d'anaplasmose en France. Rev. méd. vét., *39*, (1930): 83-85.

[19]Lotze, J. C., and Yiengst, M. J.: Mechanical Transmission of Bovine Anaplasmosis by the Horsefly *Tabanus Sulcifrons* (Macquart). Am. J. Vet. Res., *2*, (1941): 323-326.

[20]Lotze, J. C.: Carrier Cattle as a Source of Infective Material for Horsefly Transmission of Anaplasmosis. Am. J. Vet. Res., *5*, (1944): 164-165.

[21]Morris, H., Martin, J. A., and Oglesby, W. T.: An Attempt to Transmit Anaplasmosis by Biting Flies. J. A. V. M. A., *89*, (1936): 169-175.

[22]Piercy, P. L.: Fifty-first Ann. Rep., Texas Agric. Exper. Sta., (1938): 12.

[23]Piercy, P. L., and Schmidt, H.: Fifty-fourth Ann. Rep., Texas Agric. Exper. Sta., (1941): 110-111.

[24]Quevedo, J. M.: La Tristeza de los Bovinos, (Piroplasmosis, Babesiellosis, Anaplasmosis), Jesus Menendez, Edit., Buenos Aires, (1929): 192.

[25]Rees, C. W.: The Experimental Transmission of Anaplasmosis by *Rhipicephalus Sanguineus*, North Am. Vet., *11*, (1930): 17-20.

[26]Rees, C. W.: Experimental Transmission of Bovine Anaplasmosis and Piroplasmosis by Means of an Infected Lancet. North Am. Vet., *11*, (1930): 17-20.

[27]Rees, C. W.: The Experimental Transmission of Anaplasmosis by *Dermacentor Variabilis*. Science, *75*, (1932): 318-320.

[28]Rees, C. W.: The Experimental Transmission of Anaplasmosis by *Dermacentor Andersoni*. Parasitology, *24*, (1932): 569-573.

[29]Rees, C. W.: Transmission of Anaplasmosis by Various Species of Ticks. U. S. Dept. of Agric., Tech. Bull. 418, Washington, D. C., 1934.

[30]Rees, C. W., and Avery, J. L.: Experiments on the Hereditary Transmission of Anaplasmosis by Ticks. North Am. Vet., *20*, (1939): 35-36.

[31]Rosenbusch, F., and Gonzales, R.: Die Tristeza Uebertragung durch Zecken und dessen Immunitätsprobleme. Arch. f. Protistenk., *58*, (1927): 300-320.

[32]Rozeboom, L. E., Stiles, G. W., and Moe, L. H.:

Anaplasmosis Transmission by *Dermacentor Andersoni* Stiles. J. Parasitol., **26**, (1940): 95-100.

[65]Sanborn, C. E.: Anaplasmosis Investigations: Rep. Oklahoma A. and M. Coll. and Agric. Exper. Sta., 1926-1930, (1931): 250-254.

[66]Sanborn, C. E., Stiles, G. W., Moe, L. H., and Orr, H. W.: Anaplasmosis Investigations. Rep. Oklahoma A. and M. Coll. and Agric. Exper. Sta., 1926-1930, (1931): 254-264.

[67]Sanborn, C. E., Stiles, G. W., and Moe, L. H.: Anaplasmosis Investigations. Rep. Oklahoma A. and M. Coll. and Agric. Exper. Sta., 1930-1932, (1932): 248-250.

[68]Sanborn, C. E., Stiles, G. W., and Moe, L. H.: Preliminary Experiments in the Transmission of Anaplasmosis by Horseflies. Oklahoma Agric. Exper. Sta., Bull. 204, (1932): 1-16.

[69]Sanborn, C. E., and Moe, L. H.: Anaplasmosis Investigations. Rep. of the Oklahoma A. and M. Coll. and Agric. Exper. Sta., 1932-1934, (1934): 275-279.

[70]Sanborn, C. E., Stiles, G. W., and Moe, L. H.: Anaplasmosis Transmission by Naturally Infected *Dermacentor Andersoni* Male and Female Ticks. North Am. Vet., **19**, (1938): 31-33.

[71]Sanders, D. A.: Notes on the Experimental Transmission of Bovine Anaplasmosis in Florida. J.A.V.M.A., **86**, (1935): 799-805.

[72]Schmidt, H., and Piercy, F. L.: Fiftieth Ann. Rep., Texas Agric. Exper. Sta., (1937): 12-14.

[73]Sergent, Edm. Donatien, A., Parrot, L., Lestoquard, F., Plantureux, Edm., and Rougebief, H. Les Piroplasmoses Bovines d'Algerie. Arch. Inst. Pasteur d'Algérie, **2**, (1924): 1-146.

[74]Sergent, Edm., Donatien, A., Parrot, L., et Lestoquard, F. (In Memoriam): Études sur les Piroplasmoses Bovines. Inst. Pasteur d'Algérie, Alger, (1945): 816.

[75]Stiles, G. W.: Mechanical Transmission of Anaplasmosis by Unclean Instruments. North Am. Vet., **17**, (1936): 39-41.

[76]Taylor, E. L.: An Attempt to Transmit Anaplasmosis by British Biting Flies. Vet. J., **91**, (1935): 4-11.

[77]Theiler, A.: Uebertragung der Anaplasmosis mittels Zecken. Ztschr. f. Infektionskr., **12**, (1912): 105-116.

[78]Velasquez, Jose Q.: Contribucion al Estudio de las Piroplasmosis de los animales domesticos en Colombia. Rev. Med. Vet., **8**, (1938): 67-106.

[79]Zeller, H., and Helm, R.: Versuche zur Frage der Uebertragbarkeit des Texasfiebers auf Deutsche Rinder durch die bei uns vorkommenden Zecken *Ixodes Ricinus* und *Haemaphysalis Punctata Cinnabarina*. Berl. tierärztl. Wchnschr., **39**, (1923): 1-4.

Infectious Keratitis of Cattle—A Preliminary Report

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INFECTIOUS KERATITIS, or pinkeye, is one of the most important cattle diseases from an economic standpoint. All ages and breeds are susceptible. The disease is widespread throughout the country and is especially prevalent in the Midwest. The disease is more prevalent some years than others; and the well-fed, well-conditioned animal is apparently just as susceptible as the poorly conditioned one. The disease usually becomes prevalent in late spring or early summer and does not lessen in extent until frost or late fall. In some localities, keratitis is the deciding factor between profit and loss, especially in range cattle. Dairy cows lose in milk production, and range cattle lose weight rapidly.

The infection may be seen in one or simultaneously in both eyes. The lesions are confined to the cornea and conjunctiva. In every case of keratitis, the cornea attains a cloudy or milky white opacity.

The housefly, *Musca domestica*, and possibly other flies play an important part in the transmission of the disease during summer months.

HISTORY

Billings[1] observed cases of keratitis among cattle in Nebraska over sixty years ago, and gave a good clinical description of the disease. He stated that keratitis was not a new disease, and that it could be prevented from spreading through the herd by isolation and simple therapeutic treatment.

Poels[2] described keratitis among cattle in Holland. He isolated *Bacillus pyogenes* and reproduced the disease by injecting cultures of this organism between the layers of the cornea. He concluded that *B. pyogenes* was the cause of keratitis in cattle in Holland.

Allen[3] stated that keratitis had been reported in India, Holland, South Africa, eastern and western provinces of Canada, and in the United States. He called particular attention to a smoky or hazy appearance of the cornea with marked lacrimation and photophobia. He isolated a short, thick diplobacillus from eye secretions of typical cases, but could not reproduce the disease by instillation of this culture into the eyes of a calf.

Morax[4] isolated a diplobacillus from cases of conjunctivitis in man. This organism possessed most of the characteristics of the gram-negative diplobacillus isolated by Poels,[2] Allen,[3] and later by Jones and Little.[5]

Jones and Little[5] observed a disease among cattle soon after arrival from Ohio, which they named "infectious ophthalmia." The disease was readily transmitted to other cattle by contact. The disease was characterized by sudden onset, marked photophobia, and impairment of vision. The typical smoky, hazy appearance of the cornea, observed by Allen[3] in typical keratitis, was absent in most of the case of infectious ophthalmia studied by Jones and Little.[5] They stated further that lacrimation was excessive and accompanied by considerable thick, yellow discharge, which gathered at the inner canthus. The exudate flowed down over the face, and conjunctivitis and injection of the vessels of the eyeball was noted. They isolated a gram-negative diplobacillus morphologically indistinguishable from the *Bacillus morax-axenfeld.*[4] Bouillon suspensions of pure cultures, placed beneath the eyelids of normal cattle, produced characteristic inflammations.

Farley[6] found keratitis prevalent among cattle of different ages and breeds in Kansas. The disease, described as having mild, acute, and chronic types, was transmitted with fair consistency, provided virulent eye secretions were dropped on the surface of the cornea. It was not possible to reproduce the disease with bacteria isolated from active cases. Filtrates prepared from active cases of the disease would not reproduce keratitis in susceptible cattle. It was proved that a calf apparently recovered from a chronic type of infection was a source of keratitis seven months later. The disease was apparently a specific disease of cattle. Sheep, swine, rabbits, and guinea pigs could not be infected.

Baldwin[7] made bacteriologic studies of eye secretions from 93 head of cattle sent in from Montana, California, and Ohio, and isolated *Hemophilus bovis* in 93 of 112 infected eyes. This organism was identical to that isolated and previously described by Jones and Little[5] as being associated with infectious ophthalmia. These cultures, when introduced into the conjunctival sac, produced keratitis in 12 of 15 animals.

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EXPERIMENTAL

Since the work of Jones and Little[1] who identified a hemolytic diplobacillus in connection with infectious ophthalmia, and of Baldwin[2] who definitely established that *H. bovis* was responsible for causing keratitis in cattle, an important avenue of attack on the disease has been established. Research studies with keratitis were started by the Oklahoma Veterinary Research Institute at the Oklahoma A. & M. College, Stillwater, Okla., in 1947. Working facilities have been set up there and at a field laboratory in Osage County, Okla. The latter is in the heart of some of the best cattle range in the Southwest. Keratitis has been a problem in this area since the disease was first recognized. Equipment, personnel, and sufficient numbers of diseased cattle have been available for experimental purposes at all times. From Dr. Baldwin, we obtained three cultures of *H. bovis* for use in our studies.

Fig. 1—Hereford heifer affected with acute keratitis, six days after exposure with virulent eye secretions obtained from field case. Symptoms: photophobia, lacrimation, congestion of corneal blood vessels, conjunctivitis, and complete opacity of cornea with temporary blindness.

Experiment 1.—Five cows and 4 calves were exposed with 24-hour broth cultures of *H. bovis*, to which was added 5 per cent horse serum. The animals were held in screened, fly-proof stalls during the experiment. Exposures were made by dropping cultures of *H. bovis* liberally over the surface of the eye, under the eyelids, and beneath the membrana nictitans of both eyes in each animal. The exposures were continued at 24-hour intervals for six days. All exposed eyes were normal after three weeks. It was assumed that the cattle were naturally resistant or that the animals were held under environmental conditions not conducive to *H. bovis*. Therefore, eye secretions were collected from keratitis-infected calves in a large herd of range cattle and used to expose the cattle that had been previously exposed with *H. bovis*. Keratitis infection was just beginning to spread through the herd from which the infective material was collected, but only calves were infected at the time. This material produced typical cases of keratitis in 3 of the 9 animals mentioned above. Unilateral infection was produced in each of 3 calves. Exposures were made at 24-hour intervals and in the same manner as that used for *H. bovis*. The disease developed three days after the first exposures with virulent eye secretions.

Experiment 2.—Six adult rabbits and 6 guinea pigs each received exposures with 24-hour cultures of *H. bovis*, washed from blood agar slants. The cornea was not scarified in any instance. All were normal two weeks after the fifth exposure made at 24-hour intervals. Previous work by Farley[4] indicated that sheep, swine, rabbits, and guinea pigs could not be infected with virulent eye secretions from keratitis cattle. Allen[3] could not reproduce keratitis in rabbits and guinea pigs.

Experiment 3.—Twenty-four-hour cultures of *H. bovis* were washed from the surface of blood agar with physiologic saline, and one drop of this material was used to expose 10- and 12-day-old chicken embryos. When the culture was deposited on the inner shell membrane, the eggs hatched normally but, when the injections were made beneath the inner shell membrane, the embryos invariably failed to hatch. *Hemophilus bovis* could not be recovered from the inoculated eggs after forty-eight and seventy-two hours. Additional study is needed to determine the pathogenicity of *H. bovis* for chicken embryos or the ability of the organism to remain viable after 48- and 72-hour incubation in the embryonated egg.

Experiment 4.—In experiment 1, cultures of *H. bovis* could not be caused to produce keratitis in any of the 9 cattle that were held in fly-proof stalls, free from sunlight. Three of the 9 were later proved susceptible to the disease when exposed with eye secretions obtained from natural outbreaks. Therefore, a more extensive experiment using range cattle was set up at the Oklahoma Veterinary Research Institute Field Laboratory at Pawhuska, Okla. Facilities at Pawhuska were ideal for study of the disease under range conditions.

Twenty Hereford calves, approximately 6 months of age, were obtained from herds where keratitis had not appeared the year before and where there was reasonable certainty the calves had not become infected previously and recovered. The calves were divided into two lots of 10 each. Because of the limited space in the isolation area, it was necessary to complete the study of the two lots separately. Therefore, lot 1 was completed and removed from the premises before lot 2 was brought in. In each lot, the calves were fed together and were allowed to graze over a 35-acre pasture during the experiment. Both lots were allowed full sun and the shade of two small trees, if desired.

The 10 calves in lot 1 were exposed with 24-hour cultures of *H. bovis* at 24- and 48-hour intervals until six exposures had been made. Three weeks after the sixth and last exposure, all of the calves were normal. All 10 calves in lot 1 were then exposed with virulent secretions twenty-one days after the last exposure with *H. bovis*.

Lot 2 was treated in the same manner as lot 1, with the exception that two weeks after the last exposure with *H. bovis* all eyes were normal. In lot 2, exposures with virulent secretions were started the fourteenth day after the last exposure with *H. bovis*.

Three calves in lot 1 and 2 in lot 2 developed keratitis within three days after exposure with virulent secretions collected from field cases of the disease.

No attempt was made to infect the opposite eye in the infected calves, since previous work by Farley[6] showed that cattle recovered from acute or chronic infections in one eye remained susceptible in the opposite eye.

There is a wide variation in the susceptibility of calves to keratitis, but it is reasonable to expect that 1 or more of the 34 head of cattle that were exposed with *H. bovis* should have developed the disease.

Experiment 5.—Since the original laboratory cultures of *H. bovis* could not be caused to produce keratitis, fresh cultures were isolated from field cases in the locality and were used to expose susceptible cattle. It was not difficult to isolate *H. bovis* from acute cases. Infected secretions placed in serum broth for twenty-four hours and used to inoculate blood agar plates caused abundant growth of *H. bovis*. Five calves, held under range conditions and exposed with *H. bovis* cultures isolated from field cases, would not develop keratitis, even though numerous exposures were made using 24-hour cultures in each case. No attempt was made to check the 5 calves for susceptibility to the causative agent present in active field cases because inclement weather caused a cessation of these studies.

DISCUSSION

From observations in the course of the experiments reported above, and previous work by Farley, it appears that the following generalizations can be made about this disease. It is likely that *H. bovis* is not the primary factor in the production of keratitis but only a secondary invader along with other bacteria that have been isolated previously from diseased and normal eyes. However, it will be necessary to make further studies before *H. bovis* is entirely eliminated as a possible etiologic factor. An immunizing agent is needed to protect cattle from this disease.

Morphology and Cultural Characteristics.—The *H. bovis* cultures supplied by Baldwin were found by the authors to meet the description of the bacterium as described by Jones and Little,[5] and later by Baldwin.[7] The organism was a gram-negative rod, measuring 1.5 to 2.0 μ in length and 0.5 to near 1.0 μ in width. Pleomorphism was present in old cultures. However, chains of four to eight bacilli were observed on blood agar plates and slants. The organism was nonmotile and did not form spores. Dextrin, dextrose, dulcitol inulin, lactose, maltose, mannitol, salicin, sucrose, and xylose were not fermented, and indol was not formed. Culture transfers to agar that contained 2 to 5 per cent horse or bovine blood after a 24-hour incubation produced characteristic growth for this organism.

Abundant growth was obtained on blood agar after twenty-four hours. The colonies were round, greyish white, and translucent, with a narrow zone of hemolysis. The hemolytic zone increased to approximately 3 to 4 mm. after forty-eight hours. Later, the colonies became fragile. Cultures would also grow on plain agar, but less abundantly. Other characteristics were inability to produce indol and failure to reduce nitrates to nitrites.

Bacteriology.—It was interesting to note that in some clinical cases, the cloudy cornea developed a small protrusion, usually at a point just below the center of the eye. This elevation of the cornea suggested a small break in the cornea that might be due to pressure from within the eyeball. The eyeball was removed surgically and cultures made from the raised area. Myriads of gram-negative diplobacillus were isolated. It was noted that this elevation was not due to pressure within the anterior eye chamber, but to changes between the layers of the stratified epithelium of the cornea. When the upper layers of the cornea were carefully dissected away, the blister or raised area disappeared and the lower layers presented a cloudy but otherwise normal

appearance. The anterior eye chamber was cultured by introducing a Pasteur pipette or a sterile hypodermic needle through the cornea and extracting some of the vitreous humor. In each instance, where the cornea was not ruptured, the interior of the eye was found to be sterile.

Pathology.—The authors observed that the lesions of keratitis depend upon whether the disease is mild, acute, or chronic.

Fig. 2—A 6-month-old Hereford-Shorthorn cross, affected with acute keratitis, five days after exposure with virulent eye secretions. Symptoms: photophobia, lacrimation, congestion of circumcorneal blood vessels, conjunctivitis, cloudiness of cornea with heavy concentration of leucocytes near center of cornea, and blindness.

Mild Infection.—Lacrimation, conjunctivitis, and slight cloudiness of the cornea are always present in this type of infection. There is very little distention of circumcorneal blood vessels and the eye tissues tend to clear up within a few days, provided the infected animal is isolated in a stall away from sunlight, flies, and dust.

Acute Infection.—This type is characterized by its sudden onset and rapid progress. Lacrimation, photophobia, and cloudiness of the cornea, which in some instances may cover the entire cornea, with impairment of vision resulting in blindness, are observed in some cases. A thick mucous secretion flows continuously over the face. Circumcorneal blood vessels are congested and distended. If the disease is observed in its early stages, a white or grey spot can be seen just below the center of the eye.

The conjunctiva and membrana nictitans show reddening, swelling, and congestion in conjunction with opacity of the cornea. This type of infection can be checked, to a great extent, by isolating the affected animal in a dark stall free from flies and dust. The affected cow, when handled in this manner, usually recovers satisfactorily without medicinal treatment.

Chronic Infection.—The difference between acute and chronic infections lies in the extensive changes in the cornea. Thickening of the cornea, ulceration, and hypopyon develop, depending upon whether the cornea ruptures and allows the anterior eye chamber to become involved. The membrana nictitans becomes congested and protrudes. The excessive infiltration of the cornea with leucocytes usually results in impairment of vision or temporary blindness. A reddish-to-yellow discoloration of the cornea is usually observed in these cases. The lens may escape, should a break appear in the cornea. This type of infection is often observed among range cattle that are continually exposed to sunlight, wind, dust, flies, and other conditions common to this type of cattle. Chronic infection may be prevented from developing, in most cases, if mild or acute cases are isolated and placed in darkened stalls.

SYMPTOMS

Keratitis usually appears in the herd without warning and it may be difficult or impossible to trace the origin of the infection. Different types of infection were observed among animals in the same herd, which indicates a wide variation in resistance and susceptibility of cattle to the disease. The disease affects all ages and breeds but is confined principally to young cattle. Photophobia, lacrimation, cloudiness of the cornea, impairment of vision, injection of corneal blood vessels, and conjunctivitis are present in all cases. In extreme cases, there is a total blindness in one or both eyes. As the disease progresses, the animal shows continued discomfort and loss of interest in grazing. There is a rapid loss in weight, especially in hot dry weather. There is a wide variation in the time required for the cornea to lose the opacity. In some cases, the cornea remains cloudy for an indefinite period. The mortality is negligible and may be attributed to secondary conditions.

PREVALENCE

Keratitis is usually most prevalent in Oklahoma from the months of May to October. With the first frost, the infection decreases rapidly. However, the disease has been observed during December, January, and February. Infectious keratitis is equally prevalent at the time of year when carotene is abundant in the feed. Lowered resistance is apparently not a contributing factor,

since cattle that are well fed and in good condition appear to contract the disease just as readily as those in poor condition. In some herds, more than 10 per cent of the cattle may be affected at one time. With cooler weather and fewer flies, keratitis ceases to spread through the herd.

Infectious keratitis is apparently different from nutritious keratitis, but pathologic changes in eye tissues of cattle affected with the two types are similar.

TREATMENT

The cause of bovine keratitis has not been established. Until the etiologic factor is determined, it will be necessary to treat the condition symptomatically. Isolation of the actively infected animal in darkened, fly-proof stalls has proved more effective than medicinal treatment in curing the disease.

When isolation is not considered practical, medicinal treatment must be resorted to.

Sulfa-urea powder, dusted over the eye, has shown encouraging benefit. Sulfathiazole ointment is very effective, but is more difficult to use than powder or solution. These drugs have apparently proved effective as bactericides because bacteria common to keratitis are difficult to isolate from eye secretions for three to five days following treatment.

Penicillin has been used in a few experimental cases. Its effectiveness has not been determined.

Other medicinal treatment, used for years, includes boric acid solution, argyrol, silver nitrate, calomel, and mercurochrome.

SUMMARY

1) Keratitis is an infectious disease peculiar to cattle, and the primary lesions are confined to the cornea and conjunctiva.

2) Cultures of *Hemophilus bovis* (Baldwin) would not produce keratitis in 34 head of cattle which remained normal following numerous exposures with 24-hour cultures of this organism.

3) Cultures of *H. bovis*, isolated from active field cases, would not reproduce the disease in 5 normal calves.

4) Rabbits and guinea pigs failed to develop diseased eyes following numerous exposures with *H. bovis*.

5) It appears that *H. bovis* is a secondary invader in keratitis along with other microörganisms present in eye secretions obtained from active cases.

6) Twenty-four-hour cultures of *H. bovis* caused destruction of 10- and 12-day-old chicken embryos, provided the culture was injected beneath the inner shell membrane. Cultures injected outside of the inner shell membrane did not interfere with hatching.

7) The disease is treated symptomatically by most veterinarians and stockmen.

References

[1]Billings, Frank S.: Keratitis Contagiosa in Cattle. Bull. Nebraska Agric. Exper. Sta., June, 1889.

[2]Poels, J.: Keratitis infectiosa der runderen. (Keratitis pyobacillosa). Tijdschr. voor veeartsenijkunde, *38*, (1911):758-766.

[3]Allen, J. A.: A Preliminary Note on Infectious Keratitis. J. A. V. M. A., *54*, (1918-19):307-313.

[4]Morax, Victor. Note sur un diplobacille pathogene pour la conjonctivite humaine. Inst. Pasteur, Paris. Annales, *10*, (1896):337-345.

[5]Jones, F. S., Little, Ralph B.: An Infectious Ophthalmia of Cattle. J. Expti. Med., *38*, (1923): 139-148.

[6]Farley, H.: Keratitis. Vet. Student, *3*, (1940):74.

[7]Baldwin, Earl, M., Jr.: A Study of Bovine Infectious Keratitis. Am. J. Vet. Res., *6*, (1945):180-187.

Further Studies on Hemolytic Icterus in Foals

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THIS ARTICLE deals with the results obtained in studying 22 sensitized mares from June, 1948, to June, 1949, and with investigations designed to prevent the development of hemolytic icterus in newborn foals. The treatment of affected foals also was considered.

In discussing the cause of death in foals which were bacteriologically negative, Dimock, Edwards, and Bruner[1] suggested that a certain type of fatal jaundice resembled erythroblastosis fetalis in human infants. A study of 6 icteric foals by Bruner, Hull, Edwards, and Doll[2] showed in each case that the erythrocytes of the stallion were agglutinated by the serum of the mare and that the red cells of a surviving foal reacted like those of its sire. Bruner, Hull, and Doll[3] demonstrated that cases of hemolytic icterus occurred in newborn foals when mares sensitized to certain types of erythrocytes were bred to stallions which transmitted that type of blood cell to their offspring. The disease appeared in an apparently normal newborn foal after it had absorbed the erythrocyte-destroying antibodies from the colostrum of its dam (Bruner, Edwards and Doll[4]). The laboratory methods used in diagnosing hemolytic icterus in newborn foals were discussed by Bruner.[5]

While the studies on the relation of blood factors to icterus in foals were being conducted, Caroli and Bessis,[6] and Bessis and Caroli[7] called attention to the involvement of blood factors in icterus of newborn mules. Bruner, Brown, Hull, and Kinkaid[8] showed that hemolytic anemia could be produced in baby pigs by injecting gestating gilts with erythrocytes of the boar to which they were bred.

MATERIALS AND METHODS

Nine foals were fed mare serum having a titer of 1 : 10,000 against *Salmonella abortivoequina*. Each foal received 1,000 cc. of serum in a single feeding through a stomach tube. Of the 9 foals, 3 were less than 12 hours old, 2 were between 12 and 24, 3 were between 24 and 36, and 1 was 72 hours old at the time of feeding. Before the antiserum was administered, it was established that neither the mares nor the foals carried agglutinins in their serums for the *S. abortivoequina* antigen. Within twelve hours following the forced feeding, the 3 foals which were less than 12 hours old showed titers of 1 : 200 against the test antigen. The 2, 12- to 24-hour-old foals developed titers of 1 : 50 and 1 : 100, respectively, while the foals that had attained an age of 24 to 36 hours or more did not show any agglutinins for *S. abortivoequina* at a titer of 1 : 10. Tests made at twenty-four and forty-eight hours following the administration of the serum gave the same results. At the end of seven days, the titers of the foals which had absorbed the antibodies had dropped to about one half of their twelve-hour level.

During the 1949 foaling season, a mare that produced a hemolytic icteric foal was found to possess a hemagglutinin titer of 1 : 32 and a hemolysin titer of 1 : 1,000 when tested against the erythrocytes of the sire of the foal. Blood plasma from this sensitized mare was injected intravenously into 3 normal 1-day-old foals. These foals were selected because their red cells were not compatible with those of the mare, and each foal received a single injection of 500 cc. of plasma. Erythrocyte counts made prior to the injection showed 11,300,000; 12,180,000; and 12,100,000 per cubic millimeter, respectively. The erythrocyte count of the first foal dropped steadily from 11,300,000/cmm. until it reached 4,310,000/cmm. on the sixth day after the injection was made. On the seventh day, the count was 4,360,000/cmm. and it continued to climb until it was 10,780,000/cmm. two months later.

Erythrocytes from the second foal agglutinated *in vitro* at about 25 per cent of the titer obtained for those of the first foal. Furthermore, the hemolysin titer of the mare was 1 : 1,000 for the first foal and 1 : 100 for the second foal. The erythrocyte count of the second foal attained its lowest point of 6,090,000/cmm. on the third day following the injection. It was back to 12,490,000 within one month. The erythrocytes of the third foal were attacked by the antibodies in the mare's plasma to about the same degree observed in the first foal. Its count dropped steadily from 12,100,000/cmm. down to 5,180,000/cmm. the sixth day after the injection was given. On the seventh day, it was 6,000,000 and, within two months, it had climbed to 11,800,000/cmm. It was demonstrated that a newborn foal whose

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erythrocytes were compatible with those of its sensitized dam could safely nurse the dam's colostrum. Even though the colostrum possessed a high hemagglutinin titer, and the foal acquired antibodies against the type of erythrocytes which had sensitized the mare, its own red cells were not attacked.

In two instances, a 5-day-old foal was allowed to suck the colostrum from a sensitized mare. Both mares had hemagglutinin titers of 1 : 64 in their colostrums, but after nursing no detectable agglutinins could be demonstrated in the blood of either foal. It also was noted that hand milking a mare at one-hour intervals for twenty-four hours would reduce the hemagglutinin content of the milk from a titer of 1 : 64 to a titer of less than 1 : 2.

Twenty sensitized mares were under supervision during 1949. Ten of these mares were barren and 2 of the pregnant mares aborted because of equine virus-abortion infection. Eight mares produced normal foals, which are alive at this writing. In studying the sensitized mares, a blood sample was drawn from each mare during the sixth to eighth month of gestation. Samples from the barren mares also were included. Each sample was tested with the type of erythrocytes to which the mare was sensitized and the samples from the pregnant mares also were tested against the erythrocytes of the stallions to which they were bred. It was determined that no mare carried a hemagglutinin titer of more than 1 : 8, even though some were mated with stallions whose red blood cells were incompatible with the mare's red cells.

Hemagglutinin titers of the barren mares were 1 : 4 or less, and no mare carried hemolysins at a titer level of 1 : 10 or higher. A second blood sample taken from each pregnant mare was examined within two weeks of the date of parturition. Of the 8 pregnant mares receiving the second test, 5 were bred to stallions carrying erythrocytes compatible with the mares' cells. Matings with these stallions were selected on the basis of erythrocyte compatibility tests and in no instance did the hemagglutinin titers of the mares increase during the gestation period. All 5 foals were allowed to nurse their dams at will and none developed hemolytic icterus.

Three of the mares were bred to stallions whose erythrocytes were incompatible with the mares' erythrocytes. In one case, the hemagglutinin titer of the mare climbed from 1 : 2 to 1 : 8 during the gestation period. At the time the foal was born, the hemagglutinin titer of the mare's colostrum was 1 : 32 and the foal was not allowed to nurse. A 5-day-old foal was put on the mare and, after it remained for fifteen hours, the hemagglutinin titer of the milk was less than 1 : 2. The offspring of the sensitized mare then was returned to its dam and did not develop hemolytic icterus. The hemagglutinin titer of the second mare climbed from 1 : 2 to 1 : 16 during gestation. A hemolytic titer of 1 : 10 also developed. In this case, the foal was withheld from its dam and the dam was milked by hand at hourly intervals for thirty-six hours. At that time, the foal was returned to its dam and was permitted to nurse for the first time. It did not develop hemolytic icterus, nor acquire a detectable hemagglutinin titer. The hemagglutinin titer of the third mare remained at a level of 1 : 8 throughout the gestation period. This mare was sensitized the previous year by intravenous injections of the erythrocytes of its mate. A hemolytic icteric foal was produced (Bruner, Hull, and Doll[3]) which recovered without treatment. The mare again was bred to the same stallion and, in 1949, foaled a normal colt, which developed a slight amount of icterus and showed a red cell count of 5,960,000/cmm. on the third day after birth. This colt recovered without treatment.

During the 1949 foaling season, 10 additional mares produced hemolytic icteric foals for the first time. Within the past two years, 19 cases of icterus were seen in mares previously not known to be sensitized. Accordingly, the known number of sensitized mares has more than doubled since 1947. Of the 10 new cases found, 2 were diagnosed on postmortem examination and subsequent blood tests, 2 died before treatment could be administered, 1 recovered without treatment, and 5 recovered following treatment. The mares that produced icteric foals carried hemolysin titers ranging from less than 1 : 10 up to 1 : 1,000 and hemagglutinin titers of 1 : 4 to 1 : 128. When symptoms of hemolytic icterus appeared in the newborn foals within twenty-four hours after birth, the hemolysin titers of their dams were 1 : 100 or higher. Foals produced by sensitized mares bearing hemolysin titers less than 1 : 10 developed marked icterus and weakness as late as the sixth day following birth. It also was noted that a sensitized mare did not necessarily develop hemolysins and hemagglutinins to the same degree, but the rapidity of onset and severity of the hemolytic icterus were modified by the concentration of hemolysins.

The 5 foals which were saved all showed red cell counts of less than 4,000,000/cmm. when first observed. They were weak, icteric, and refused to nurse. They apparently were normal at birth but became ill within twenty-four to 120 hours afterwards. In each case, the sick foal was removed from its dam and given a blood transfusion. In 1 case, the foal given transfusion was placed on a nurse mare, but the other 4 were returned to their respective dams after being separated in each case for forty-eight hours. The usual transfusion consisted of removing 500 to 1,000 cc. of blood and

replacing it with 1,000 to 2,000 cc. of compatible blood. In some instances, an additional 500 cc. was administered about twelve hours later. In selecting a donor for a hemolytic icteric foal, it is necessary to remember that the foal is carrying the erythrocyte-destroying antibodies generated by its dam and that the red cells of the donor should be matched against the serum of the dam. That is, the erythrocytes of the donor should be compatible with those of the dam. The antibodies in the serum of the icteric foal are absorbed to the extent that they do not present a true agglutination titer when tested with the donor's erythrocytes.

During the periods the icteric foals were withheld from their dams, they were fed milk from other mares, Nestle's baby food, condensed milk, or fortified cow's milk. Frequently, penicillin and/or streptomycin injections were given to ward off bacterial infections.

DISCUSSION

The feeding experiment in which *S. abortivoequina* antiserum was fed to newborn foals indicated that a foal loses the ability to absorb antibodies from its digestive tract when approximately 36 hours old. In tests where 5-day-old foals were permitted to suck mare colostrum carrying high antibody titers, it was established that they did not absorb the antibodies. This early loss of ability to absorb antibodies from the digestive tract is important because it means that the newborn foal which has inherited red cells incompatible with those of its dam can nurse its sensitized dam after being withheld for the critical period of thirty-six hours. Actually, two factors are at work to make this possible. Hand milking the mare at hourly intervals markedly reduces the antibody concentration in the milk and the foal is no longer able to absorb the antibodies. This cessation of antibody absorption may be related to the development of digestive processes in the newborn foal.

Although it was demonstrated that the foal soon was unable to absorb antibodies from its alimentary tract, the fact that many hemolytic icteric foals did not develop symptoms of the disease until four to six days after birth needed explaining. When 500 cc. of high-titered serum from a sensitized mare was injected directly into the vein of a susceptible foal, six days were required to lower the foal's erythrocyte count to the lowest level attained. Icterus was not observed in the sclera of the foal until the third day, and the most marked respiratory distress appeared on the fifth and sixth days following the injection. The same serum injected into a foal whose red cells were more refractory to the serum *in vitro* also was less active *in vivo* and reached the peak of its erythrocyte-destroying activity on the third day. Red cell counts of normal newborn Thoroughbred foals were found to be 10,000,000 to 12,000,000/cmm. on the first day of life. In some instances, these counts dropped a little below 10,000,000/cmm. during the first week of life, but usually fell no lower.

The diagnosis of hemolytic icterus in a foal depends upon finding specific erythrocyte-destroying antibodies in the blood of the dam, as well as icterus, weakness, a low erythrocyte count, yellowish red serum, and agglutinated red cells in the foal. Certain cases of *Shigella equuli* also may appear listless, weak, and icteric, and refuse to nurse. These foals sometimes show a blood count of about one-half the normal count. However, a blood sample from the infected foal will exhibit no evidence of clumped erythrocytes and blood serum from the mare will not agglutinate the foal's cells.

By testing the blood of the pregnant mare against the blood of its mate early in the gestation period, and also within two weeks of foaling, it not only is possible to outline the proper care for the sensitized mare but it appears that it also is possible to predict the appearance of icterus in the first offending mare. At present, this line of investigation is under consideration.

Out of six matings, in which stallions were selected for sensitized mares on the basis of compatibility tests, there were born 6 apparently normal foals. These foals were given no special care, nor were they withheld from nursing their respective dams. The possibility must be considered that rebreeding to the same stallion over a period of years will produce sensitivity. The fact that some mares have developed an antagonism to a certain type of stallion blood may mean that they can become sensitive to other types. When a sensitized mare is mated with a stallion whose erythrocytes are incompatible with the mare's cells, the mare's hemagglutinin titer rises during the gestation period and reaches its highest peak as pregnancy terminates. The mare artificially sensitized by injections of its mate's erythrocytes produced a hemolytic icteric foal, but on rebreeding to the same stallion did not exhibit a hemagglutinin titer rise during pregnancy.

Bruner, Hull, and Doll[3] reported that blood serums from sensitized mares will not agglutinate the erythrocytes of all the stallions that have sired such foals. In numerous instances, the erythrocytes of

the sires of icteric foals are compatible with those of mares which have produced icteric foals when mated with other stallions. Although the possibility of interference in these tests by blocking antibodies was suggested, the presence of such substances was not demonstrated.

A sensitized mare and a stallion which had sired icteric foals were mated because the blood test showed them to be compatible. A normal foal resulted which did not develop icterus even though it was not withheld from its dam. Furthermore, a comparison of the red cells of the foal and those of the sire showed no differences when tested against the serums of 33 sensitized mares. This is further argument against the existence of an Rh-like factor as the cause of hemolytic icterus in newborn foals. Wiener[9] reported congenital hemolytic disease in an infant due to A-B iso-immunization and there is no reason to assume that a similar condition does not exist in horses. It appears that certain mares become sensitized to a type of blood cells and, whenever they are bred to a stallion capable of transmitting that blood type to its offspring, a hemolytic icteric foal results.

CONCLUSIONS

Between the ages of twenty-four and thirty-six hours, a newborn foal loses the ability to absorb antibodies from horse serum placed in its stomach. Intravenous injections of high-titered serum from a sensitized mare into day-old foals which were selected by compatibility tests resulted in the production of hemolytic icteric foals that exhibited the severest anemias on the third to the sixth day after the injections. By withholding potential icteric foal cases from their dams' milk and hand milking the sensitized mares at hourly intervals for thirty-six hours, it was possible to prevent the development of icterus in the foals upon returning them to their dams. Mares bred to stallions whose erythrocytes were compatible with the mares' erythrocytes did not produce icteric foals. Cases of hemolytic icterus in newborn foals were treated successfully by removing the foal from its dam, tranfusing it with blood from a compatible donor, and returning it to the dam after forty-eight hours.

References

[1]Dimock, W. W., Edwards, P. R., and Bruner, D. W.: Infections Observed in Equine Fetuses and Foals. Cornell Vet., *37*, (1947): 89-99.

[2]Bruner, D. W., Hull, F. E., Edwards, P. R., and Doll, E. R.: Icteric Foals. J.A.V.M.A., *112*, (1948): 440-441.

[3]Bruner, D. W., Hull, F. E., and Doll, E. R.: The Relation of Blood Factors to Icterus in Foals. Am. J. Vet., Res., *9*, (1948): 237-242.

[4]Bruner, D. W., Edwards, P. R., and Doll, E. R.: Passive Immunity in the Newborn Foal. Cornell Vet., *38*, (1948): 363-366.

[5]Bruner, D. W.: Laboratory Diagnosis of Hemolytic Icterus in Foals. Cornell Vet., *40*, (1950) : 11-16.

[6]Caroli, J., and Bessis, M.: Sur la cause et le traitement de l'ictère grave des Muletons nouveaunés. Compt. rend. Acad. sci., *224*, (1947): 969-971.

[7]Bessis, M., and Caroli, J.: Anticorps anti-Mulets incomplets et bloquants chez la Jument mère de muleton ictérique. Compt. rend. Soc. de biol., *141*, (1947): 387.

[8]Bruner, D. W., Brown, R. G., Hull, F. E., and Kinkaid, Alice S.: Blood Factors and Baby Pig Anemia. J.A.V.M.A., *115*, (1949): 94-96.

[9]Wiener, A. S.: Recent Advances in Knowledge of the Rh Blood Factor, with Special Reference to the Clinical Applications. Lab. Digest, *10*, (1946): 1-12.

Critical Tests with Sodium Fluoride as an Anthelmintic for Ascariasis in Horses

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A SERIES of dilution egg-count and critical tests with 7 horses conducted by Habermann, Enzie, and Foster[1] indicated that sodium fluoride (NaF) had anthelmintic activity against the horse ascarid, *Parascaris equorum*. The present drug of choice for treatment of ascariasis in Thoroughbred horses in central Kentucky, carbon bisulfide (CS_2), performs efficiently against adult ascarids, but is not as efficient against immature forms in the small intestine. The hazard of introducing such a volatile substance into horses has been reduced by adoption of stomach tube techniques. The employment of CS_2 for treatment of the age group of horses most heavily infected by ascarids, sucklings, has been limited by difficulty attendant with drawing the animals before treatment. The efficiency of CS_2 is impaired by the presence of ingesta in the digestive tract.

The present paper presents data obtained from 20 critical tests with NaF against ascarid infection in suckling, weanling, and yearling horses.

Materials and Methods

The majority of the horses used in the critical tests were Thoroughbreds, although a few were light-boned grade horses. They ranged in age from 1 month to fully mature horses; the majority were weanlings and yearlings. Most of the horses harbored numerous roundworm species in addition to ascarids; all infections were naturally acquired.

The NaF was dissolved in tap water and administered by means of a stomach tube. The horses were on full feed when medicated and were sacrificed twenty-four hours after treatment. They were sacrificed at this time in order that worms killed and expelled from the small intestine would not disintegrate before being recorded.

At autopsy, a complete examination was made of the stomach and small and large intestines, including the rectum. Worms present were collected and counted. At autopsy, the digestive and other organs were examined for gross evidence of pathologic changes.

Preliminary investigation having indicated a tolerance limit of 5.0 Gm. per hundredweight, the critical tests were largely conducted with NaF administered at a rate of 2.5 and 1.5 Gm. per hundredweight.

TABLE 1—Tests with Sodium Fluoride (NaF) at 2.5 Gm. per Hundredweight

Horse	Weight (lb.)	Amount NaF (Gm.)	Specimens of ascarids recovered: Worms in small int.	Worms expelled from small int.	Expulsion efficiency (%)	Dead worms recovered	Live worms recovered	Ascaricide efficiency (%)
1	500	12.50	0	106	100.0	106	0	100.0
2	380	9.50	0	123	100.0	123	0	100.0
3	550	13.75	0	280	100.0	280	0	100.0
4	232	5.75	1	122	99.2	87	36	70.7
5	810	20.20	0	27	100.0	27	0	100.0
6	340	10.00	0	321	100.0	236	85	73.5
7	500	12.50	0	128	100.0	128	0	100.0
8	642	16.00	1	3	75.0	2	1	75.0
9	592	14.80	0	24	100.0	24	0	100.0
10	672	16.80	0	20	100.0	20	0	100.0
Average	521.9	13.20	0.2	115.4	99.8	103.4	12.2	89.4

Experimental Data

Sodium Fluoride at 2.5 Gm. per Hundredweight.—These tests, involving 10 horses are recorded in table 1. Of 1,156 ascarids present in the small intestine of the 10 horses, 1,154 were expelled from the small intestine and 1,134 were dead within twenty-four hours. The percentage efficiency of expulsion ranged from 75.0 to 100.0 per cent and averaged 99.8

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per cent; the efficiency of NaF as an ascaricide ranged from 70.7 to 100.0 per cent and averaged 89.4 per cent in twenty-four hours.

The worms recovered in these tests ranged in length from 2 to nearly 12 inches.

Sodium Fluoride at 1.5 Gm. per Hundredweight (table 2).—These tests involved 10 horses and there were 1,316 ascarids present in the small intestine of the horses. A total of 811 were expelled from the small intestine by treatment and 649 of these were dead within twenty-four hours. The percentage efficiency of expulsion ranged from 2.6 to 100.0 per cent and averaged 61.7 per cent. The efficiency of NaF as an ascaricide ranged from 0.0 to 100.0 per cent and averaged 49.3 per cent. One horse, a 260-lb. suckling, given only 3.9 Gm. of NaF (table 2, horse 4), was largely responsible for its poor performance at this dosage. If this animal were considered an exception, the efficiency of NaF at 1.5 Gm. per hundredweight in expelling ascarids from the small intestine would have been 95.6 per cent and its efficiency as an ascaricide would have been 78.2 per cent.

The worms recovered in these tests ranged in length from 2 to nearly 12 inches.

DISCUSSION

When administered to horses at a rate of 2.5 Gm. per hundredweight, NaF was extremely effective against *P. equorum*, both from the standpoint of expelling worms from the small intestine and as an ascaricide. It was as effective against immature as against mature ascarids.

When administered at the rate of 1.5 Gm. per hundredweight, efficiency of expulsion and efficiency as an ascaricide were reduced at least by 3.0 and 12.0 per cent, respectively.

Judged on the basis of the above 20 critical tests, NaF appears to be of greater value than CS_2 in a control program for ascarid infection in horses, because it is more effective against immature stages. The fact that it may be administered to horses on full feed would appear to be especially advantageous in the treatment of sucklings.

A therapeutic dose of 5.0 Gm. would appear to represent the minimum dosage from which efficiency of expulsion from the small intestine and efficiency as an ascaricide can be obtained.

No effort was made to record the efficiency of NaF against tapeworms, pinworms, strongyles, or cylicostomes. The drug had no effect upon bots in the stomach.

Clinical observations, made of the 20 horses used in the critical tests and of a number of other horses used in dilution egg-count tests, indicate that NaF was generally well tolerated. Moderate gastritis and enteritis were found frequently at postmortem and were the only gross pathologic changes observed. Animals used in dilution egg-count tests frequently exhibited severe diarrhea twenty-four to forty-eight hours after treatment. Recovery in seventy-two hours was constant.

Clinical observations following drenches with NaF are exemplified by records obtained from 2 Thoroughbred mares used in tests for tolerance. The mares were given 31.5 Gm. of NaF (2.5 Gm./cwt.) by stomach tube. The animals were normal in appearance; their temperature, respiration, and pulse were in normal ranges. Twenty-four hours after treatment, both animals were depressed, off feed, and beginning to scour. Inappetence, depression, and severe scouring were evident forty-eight hours after treatment. Water consumption increased markedly. The pulse, temperature, and respiration remained unchanged. By seventy-two hours after treatment, the animals had completely recovered.

Younger and lighter animals used in dilution egg-count tests, which received

TABLE 2—Tests with Sodium Fluoride (NaF) at 1.5 Gm. per Hundredweight

Horse	Weight (lb.)	Amount NaF (Gm.)	Specimens of ascarids recovered					
			Worms in small int.	Worms expelled from small int.	Expulsion efficiency (%)	Dead worms recovered	Live worms recovered	Ascaricide efficiency (%)
1	480	7.2	0	3	100.0	0	3	0.0
2	720	10.8	0	55	100.0	55	0	100.0
3	195	2.0	2	1	33.3	1	2	33.3
4	260	3.9	479	13	2.6	4	488	0.8
5	500	7.5	2	85	97.7	77	10	88.5
6	460	6.9	0	9	100.0	9	0	100.0
7	700	10.5	0	32	100.0	32	0	100.0
8	575	8.6	0	343	100.0	229	114	66.7
9	425	6.4	14	61	81.0	34	41	45.3
10	352	5.3	8	209	96.3	208	9	95.8
Average	466.7	7.0	50.5	81.1	61.7	64.9	66.7	49.3

from 5 to 20.0 Gm. of NaF at 2.5 or 1.5 Gm. per hundredweight, generally exhibited temporary diarrhea, but rarely depression and inappetence.

Blood samples, taken at intervals before and after treatment of a yearling Thoroughbred with NaF at 1.5 Gm. per hundredweight, established the following changes in the blood picture: a temporary hemoconcentration, some hemolysis, and an evanescent leucocytosis. The packed cell volume was greatest within eight to ten hours after treatment, as was also the amount of hemoglobin. About nineteen hours after treatment, the packed cell volume and hemoglobin had initiated a return to pretreatment levels. Some hemolysis, evident in dark red plasma in the hematocrit tubes, occurred about ten hours after treatment. This hemolysis was considered related to NaF treatment and not to be the anticoagulant employed, an ammonium and potassium oxalate mixture. A temporary rise in numbers of white blood cells, as observed in the hematocrit, was considered associated with the temporary gastritis and enteritis found to develop after administration of NaF.

These two series of ten critical tests established that NaF was remarkably efficient as an ascaricide for horses and confirmed preliminary observations.[1] It was as effective against immature as against mature ascarids. Its efficiency more than offset the temporary gastritis and enteritis following treatment. The moderate diarrhea is considered desirable in prevention of possible occlusion and absorption of toxic materials liberated by dead ascarids. Because of the occasional severe diarrhea and hemolytic action on horse blood produced, which are under continuing investigation, the writers, at present, do not recommend NaF for therapeutic treatment of ascariasis in horses.

SUMMARY

Twenty critical tests were conducted with sodium fluoride (NaF), administered in a drench to horses on full feed at the rate of 2.5 Gm. per hundredweight, or 1.5 Gm. per hundredweight, as an anthelmintic against ascariasis in horses.

At 2.5 Gm. per hundredweight, the efficiency of NaF in expelling ascarids from the small intestine was 99.8 per cent, on average, and its efficiency as an ascaricide was 89.4 per cent, on average, within twenty-four hours after administration.

At 1.5 Gm. per hundredweight, efficiency of expulsion and efficiency as an ascaricide were reduced to 61.7 and 49.3 per cent, respectively.

At either rate of administration, immature ascarids were as susceptible to the action of NaF as mature ascarids.

Moderate-to-severe diarrhea, moderate gastritis and enteritis, hemoconcentration, leucocytosis, and hemolysis were observed following treatment.

References

[1]Habermann, R. T., Enzie, F. E., and Foster, A. O.: Tests with Fluorides, Especially Sodium Fluoride, as Anthelmintics for Swine. Am. J. Vet. Res., 6, (1945) 131-144.

The Reaction of Blood Cells in Experimental Listeriosis of Sheep

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THIS IS a report on a study of the reaction of circulating blood cells following exposure of sheep to *Listeria monocytogenes* by various routes.

Since the finding by Murray, Webb, and Swann[20] of a bacterium which would cause a mononuclear leucocytosis in rabbits, many investigations have been made of the disease caused by *L. monocytogenes*. The disease (listeriosis, listerellosis, or circling disease) has been recognized as of economic importance in domestic livestock even though its exact incidence is unknown. Many species of animals, including man, have been infected with *L. monocytogenes*. Adequate reviews of the early literature have been made by Graham *et al.*,[13] Gifford and Jungherr,[12] and Julianelle.[20] The study of monocytosis induced in laboratory animals with *L. monocytogenes* received impetus with Nyfeldt's[20] report of finding this organism in a human patient with infectious mononucleosis. The significance of this association is not yet clear.[20] Various reports[1, 21] have confirmed the finding[20] of monocytosis with subfatal infections in rabbits. Similar observations have been recorded for guinea pigs and mice.[20] Seastone[20] observed monocytosis in chickens. Varying reports have been made on the blood cell reaction of sheep and cattle with *L. monocytogenes* infection. Gill[13] observed a high percentage of neutrophils in field cases in sheep and a similar high percentage of neutrophils at death in 2 experimentally infected sheep. Biester and Schwarte stated that monocytosis was observed in field cases of infection in sheep and swine. [2, 3, 4] Cole's[5] data indicate a terminal leucopenia in a cow with listeriosis. Graham *et al.*[13] report several observations of the blood cells in both field cases and experimental infection in sheep, cattle, and swine. Although they did not find monocytosis, the blood picture was not clearly portrayed.

METHODS

The culture of *L. monocytogenes* strain 136 used in most trials was isolated from the brain of a sheep which died with listeriosis during an outbreak in January, 1947. It was maintained in tryptose broth or on tryptose agar mediums. A 24-hour tryptose broth culture was pathogenic for sheep, usually causing death in 0.1-ml. amounts given intracerebrally and by intracarotid injection at the rate of 0.05 ml. per pound body weight. Six of the tests were made with strain 578 *L. monocytogenes* isolated from the same outbreak as strain 136. The strains had shown similar properties and virulence.

All exposures were made with a 24-hour tryptose broth culture grown at 37 C. The amount used varied with the mode of exposure. These were: intravenous (jugular vein) and *per os*, 0.1 ml. per pound body weight; intracarotid (under local anesthesia directing injection either with or against the flow of blood), subcutaneous (region of neck or flank), intramuscular (biceps femoris muscle) and median artery, 0.05 ml. per pound body weight; and intracerebral, intradermal (region of neck), and marrow (tibia), 0.25 ml. The 28 test sheep were used in pairs for each trial. Most of them were used for more than one trial so that 63 tests were made. Six of the sheep came from a flock in which listeriosis had occurred, and 22 sheep came from the University of Nebraska breeding flock, in which no listeriosis had been observed. The sheep were adults of different ages, ranging from 8 months to 9 years old. The sheep were housed in isolation stalls throughout the trials. Observations were made on the leucocytes and the rectal temperatures. The leucocyte counts were made in duplicate. Two blood films were prepared at each observation and 200 cells counted on each blood film. The absolute values for the different types of leucocytes were calculated and the averages of the two sets were used to represent the number of cells at that time. Several observations were made prior to each exposure to establish a base line for normal values and at varying intervals after inoculation.

NOMENCLATURE

The leucocytes were classified by the usual

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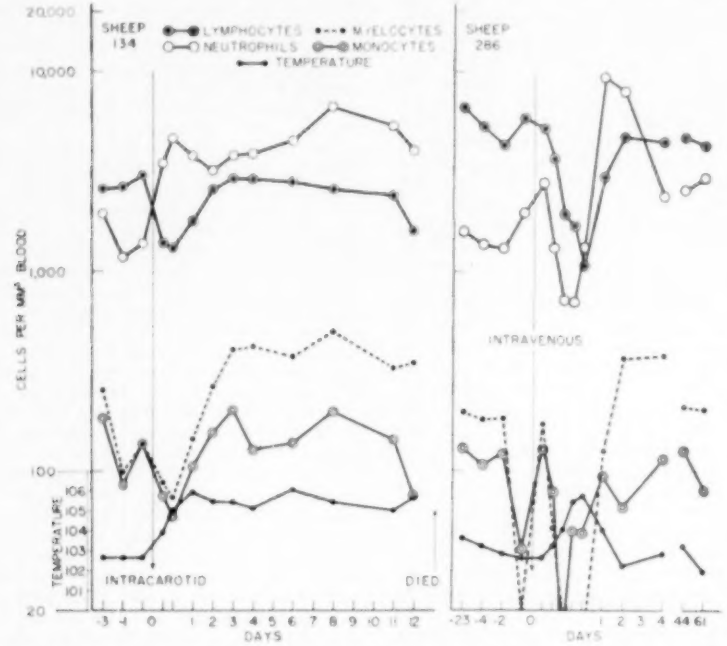
criteria into lymphocytes, monocytes, eosinophils, basophils, and neutrophils. Immature neutrophils were observed frequently. Two classifications were used for the neutrophil granulocytic series following the definitions of the committee for clarification of nomenclature.[7] The term neutrophils includes both the segmented and band cells as representing adult cells. The term myelocyte was used for all immature neutrophils and includes metamyelocytes, myelocytes, progranulocytes, and myeloblasts.

RESULTS

It became apparent, early in the work, that injection of *L. monocytogenes* caused an increase in the number of circulating neutrophils. Following the demand for neutrophils, many immature cells of this series appeared. In some instances of particularly heavy demand for neutrophils, myeloblasts were found in the circulating blood. Apparently normal sheep had immature neutrophils in the blood stream in varying numbers. These had specific neutrophilic granulation. No progranulocytes or myeloblasts were found in the blood of normal sheep. A series of developmental stages of neutrophils is shown in plate I. Ordinarily, with myelocyte counts in the range of 200 to 400 per cubic millimeter, most of the cells were of the types illustrated as 5 to 9. When high myelocyte counts of 800 to 1,000/mm.^{3} were found, cells similar to those illustrated as 1 to 4 were found, although their number was relatively small.

In order to establish the approximate

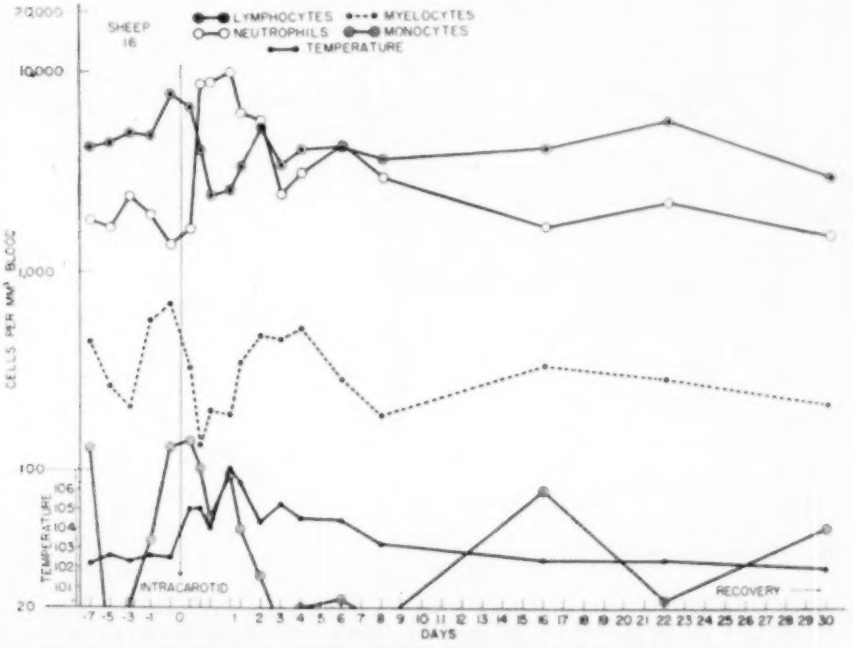


Graph 1—Leucocyte counts and temperatures on sheep before and after exposure to *Listeria monocytogenes*. Counts of leucocytes are plotted on logarithmic scale and temperature values follow arithmetic progression. Sheep 134 was given a culture of *L. monocytogenes* in the carotid artery and had no previous exposure. On the day of exposure (0), the two postexposure counts were made at two and five hours. The persistent increase in neutrophils and temporary decrease of lymphocytes with a continuously elevated temperature is typical of an experimentally produced, fatal listeriosis.

Note the increase of myelocytes following the demand for neutrophils. Monocytes vary within a broad range. Sheep 286 was given a culture into the jugular vein. On the day of exposure (0), the five postexposure counts were made at ten minutes and one, two, three and one-half, and six hours. The immediate decrease of neutrophils followed by an increase was typical for this type of exposure in previously used sheep.

normal values for the different types of leucocytes in the blood, 259 observations on 19 sheep were examined statistically. These represented from two to 53 observations on each sheep, made at a time when the sheep were considered normal. The different sheep had variations in the normal levels of the different types of leucocytes. Table 1 summarizes the findings by showing the group average as well as the high and low individual averages for the different types of leucocytes. These average values are accompanied by their standard deviations from the mean. While these values are not in exact agreement with others as reported in the literature, e.g., Prescott *et al.*,[27] they serve to indicate the normal range of the sheep actually used in these studies.

To test the effect of tryptose broth medium alone on cells of the blood and on temperature, 2 sheep were given intravenous injections in the quantities routinely used for cultures of *L. monocytogenes*. The counts for lymphocytes and body temperatures were unaltered. The neutrophils in both sheep were moderately increased (about one half) six hours after injection but had returned to normal levels the next day. Two further experiments were made on the same sheep in which the fractions of the medium were used separately. In one trial, dextrose in saline solution (concentrations as in culture medium) was injected. Again, there was no change of temperatures or in the counts of lymphocytes. The neutrophils were moderately increased (about one third) at six hours in both sheep. The neutrophil count was normal in 1 sheep at twenty-four hours and at forty-eight hours in the other sheep. In the other trial, tryptose solution (concentration as in culture medium) was injected. A moderate increase (about one third) of neutrophils was noted in both sheep at four and one-half hours, with a return to normal at



Graph 2—Leucocyte counts and temperatures on sheep 16 before and after intracarotid exposure to *Listeria monocytogenes*. Counts of leucocytes are plotted on a logarithmic scale and the temperature values follow arithmetic progression.

This sheep had received, subcutaneously, a formolized but live culture of the same organism seven days earlier. On the day of exposure (0), the three postexposure counts were made at twenty-four and thirty hours. Abnormal values for neutrophils and an elevated temperature lasted until the eighth day. Note the range of variation of monocytes.

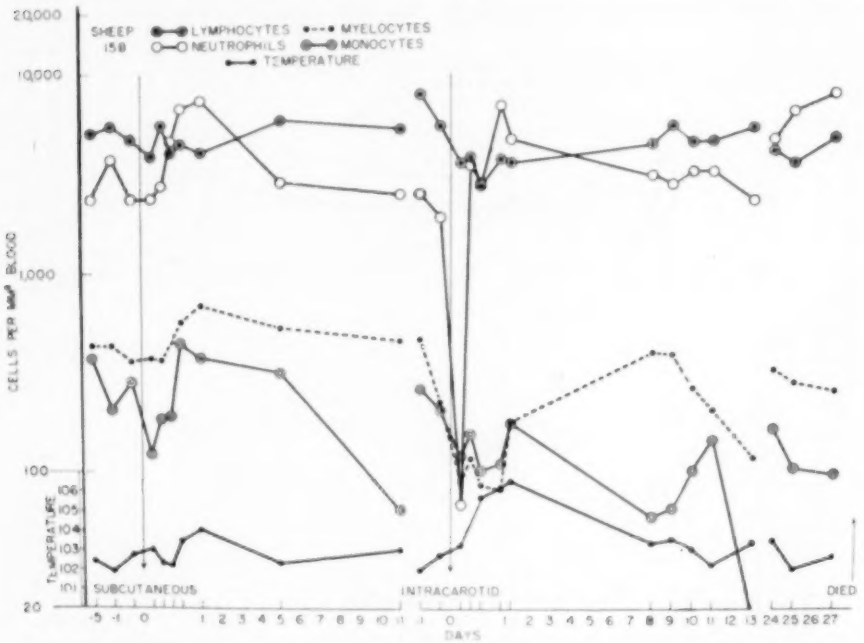
seven hours in 1 sheep and at twenty-four hours in the other sheep. There was no change of lymphocytes or body temperatures. These increases of neutrophils can be considered slight in view of the standard deviation for the mean number of neutrophils found in normal sheep (table 1), which amounted to about one fourth to one half the mean. However, since the transient change was always an increase, it has some significance. As will be noted later, the increase of neutrophils after exposure to cultures of *L. monocytogenes* was much greater than observed in these control trials.

The reactions of the cells in the blood involved lymphocytes and neutrophils and are presented in a general way for each mode of exposure. They are also summarized in table 2. Graphs of individual sheep are used to illustrate typical responses.

Intracarotid Exposure.—Eighteen trials were done in 17 sheep. Five sheep had not been previously exposed, 8 had had one previous inoculation with *L. monocytogenes* and 5 had received from six to nine previous inoculations. The 5 sheep receiving the culture for the first time died or were killed in a moribund state eight to seventeen days later. Five of the 8 sheep receiving culture via the carotid artery as their second exposure, survived and 3 died in twenty-five, twenty-seven, and fifty-seven days. Five sheep, which had had several previous exposures, survived intracarotid inoculation.

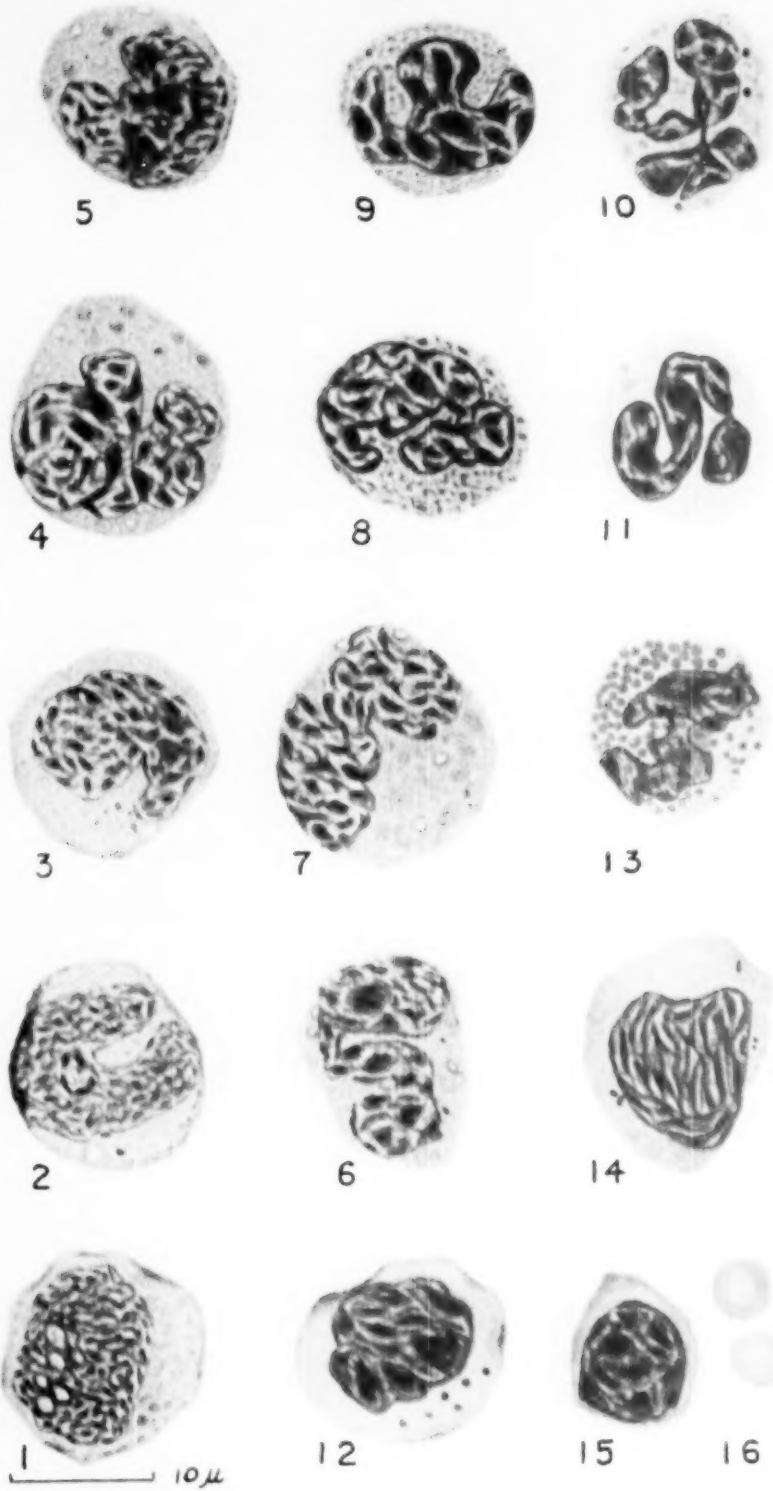
Fatal infection followed intracarotid exposure in two of five trials when injection was made against the flow of blood, and in six of 13 trials when injection was made with the flow of blood.

Following intracarotid exposure, there



Graph 3—Leucocyte counts and temperatures on sheep 158 after subcutaneous and intracarotid exposure to *Listeria monocytogenes*. There had been no previous exposure. Counts of leucocytes are plotted on a logarithmic scale and temperature values follow arithmetic progression. On the day of subcutaneous exposure (0), counts were made prior to and at ten minutes, ninety minutes, three and seven hours after injection; the next day at twenty-four and thirty hours.

An increase of neutrophils may be noted. On the day of intracarotid exposure, counts were made prior to and at ten minutes, three hours, and at six hours after injection; the next day at twenty-four and thirty hours. The low value for neutrophils at ten minutes after intracarotid exposure was an exceptional reaction. During the period from eight to twenty-four days after exposure, the counts of leucocytes and temperature values were about normal. The terminal counts of neutrophils were high.


5
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was an increase of neutrophils and a decrease of lymphocytes. These changes were marked at three hours and were associated with a rise in temperature of from 1 to 4 F. A decrease of neutrophils was noted in 1 sheep ten minutes after exposure, but 6 other sheep with counts made at ten minutes showed little or no change. The decrease of lymphocytes apparent at from three to five hours rarely lasted more than thirty hours. The peak number of neutrophils was usually noted at from seven to forty-eight hours. Lymphocytic counts were usually normal at from seventy-two to ninety-six hours.

The group of sheep not previously exposed showed similar leucocyte reactions. The record for sheep 134 is illustrated in graph 1 and is representative of the group. The neutrophil counts prior to exposure ranged from 1,175 to 1,935/mm.^{3} of blood and after exposure they ranged from 3,160 to 6,600/mm.^{3} of blood. Counts of lymphocytes were from 7,550 to 2,985/mm.^{3} of blood and dropped to 1,292/mm.^{3} of blood five hours after inoculation. The last observations were made the day before death, and a slight terminal drop of neutrophils and lymphocytes was noted. Myelocytes varied from 99 to 251/mm.^{3} of blood prior to exposure. From three to twelve days after inoculation, myelocytes ranged between 323 and 493. Monocyte counts prior to injection were from 85 to 181/mm.^{3} of blood and after exposure were within the same range (59 to 200). Rectal temperatures were from 2.5 to 3 F. above preëxposure levels until death.

The 5 sheep which had several previous exposures to *L. monocytogenes* by routes different from intracarotid were fairly uniform in their blood cell reaction. Following the initial reaction described above, the counts of different types of leucocytes returned to normal levels in from two to seven days. The rectal temperature increased from 1 to 4 F. at three to five hours and returned to normal in twenty-four to forty-eight hours.

Five sheep receiving intracarotid exposure after one previous injection of *L. monocytogenes* survived and reacted in a manner similar to that described for the 5 sheep with several previous injections, except that blood cell counts took longer to return to normal levels. The period of reaction was from three to thirty-nine days. Graph 2 illustrates the reaction of blood cells and rectal temperature of sheep 16 and was typical for the group. In this instance, the neutrophil peak of 9,840 cells/mm.^{3} of blood occurred from two to twenty-four hours after injection and, in general, remained above the normal level for eight days with some variations. Lymphocyte values dropped and returned to about normal after forty-eight hours. Temperature was elevated until the eighth day with a peak of 107 F. at twenty-four hours. Two sheep, with extended reactions of twenty and thirty-nine days, had counts which approached normal values at times. The neutrophil values reached 10,000 to 15,000 at seven to thirty hours after exposure, then dropped. In one case, neutrophils dropped to less than 1,000 at six days but usually the values were around 4,000 to 5,000. Both these sheep had elevated temperatures for two and three days, followed by four days of temperature of 1 to 1.5 F. below their previous normal level.

Three sheep receiving *L. monocytogenes* in the carotid artery after one previous exposure died twenty-five, twenty-seven, and fifty-seven days later. A curious finding was that leucocyte counts became normal for an interval after the first reaction. The data on sheep 158 are used to represent this group (graph 3). The marked drop of neutrophils ten minutes after exposure to 68 cells/mm.^{3} noted in sheep 158 was not found in any other sheep injected via the carotid artery. The later increase of neutrophils and decrease of lymphocytes was similar to that noted in other sheep with intracarotid exposure. Normal values for blood cells were found in 2 sheep at three and four days and were reached in sheep 158 between one and eight days after inoculation. The high values for neutrophils were not again noted until three days before death in sheep 158 and 1 other sheep. The other sheep of the group had an increase of neutrophils on the day of death and the day preceding. On the second and third day preceding death, neutrophils were

Legend for Color Plate on Opposite Page

Water color reproductions of cells found in circulating blood of sheep. Cells 1 to 11 represent developmental stages of neutrophils. Cell 1 is most immature and cells 10 and 11 represent adult cells.

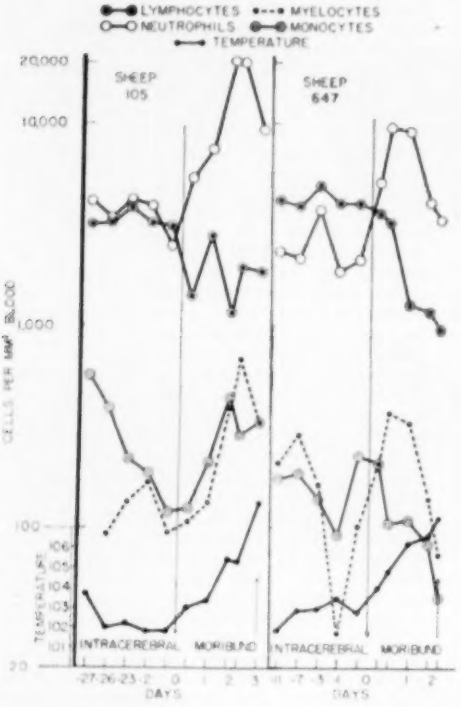
Variations in the rate of development between nucleus and cytoplasm are evident in cells 4 and 6 where the nucleus has begun to lobulate and the cytoplasm is relatively more primitive.

Cells 12 and 15 are lymphocytes; cell 13 is an eosinophil; cell 14 is a monocyte; and cells 16 are erythrocytes. Wright's stain; water color reproduction; the cell outlines were drawn with a camera lucida.

somewhat lower than normal. Only 1 of the 3 sheep had a sustained increase of temperature during the observation period. This was the animal that lived fifty-seven days and had a temperature of 104 to 105.3 F. for the last thirty days. Its normal range had been 102 to 103 F.

Intracerebral Exposure.—Eight sheep were given 0.25-ml. amounts of *L. monocytogenes* culture in either the right or left cerebral hemisphere. The operations were done with local anesthesia and under aseptic conditions. None of the sheep showed symptoms immediately after the operation that could be associated with trauma to the brain tissue. One sheep had no previous exposure to the culture and 7 had previously received *L. monocytogenes* from two to 15 times.

Six sheep died within one to five days (2 at one day, 1 at three days, 1 at four days, and 2 at five days). These animals all had essentially similar reactions of their blood cells. The data on 2 which are representative are shown in graph 4. The reaction consisted of a marked increase in neutrophils which tended to drop as the sheep were dying. Two sheep observed ten minutes after inoculation showed no change and a slight decrease of neutrophils, respectively. Lymphocyte numbers tended to drop with the lowest values at death. Monocytes and myelocytes showed no uniform change of pattern. Body tempera-



Graph 4—Leucocyte counts and temperatures on sheep receiving 0.25 ml. of broth culture of *Listeria monocytogenes* in the cerebrum. Counts of leucocytes are on a logarithmic scale and temperature values follow arithmetic progression.

Sheep 105 had no previous contact with the culture and sheep 647 had 15 previous exposures. On the day of injection (0), for sheep 105 the postexposure count was made at one and one-half hours; for sheep 647 the counts were made at three and one-half hours and six hours.

TABLE 1—Normal Values for Leucocytes per Cubic Millimeter of Blood*

	Lymphocytes		Neutrophils		Myelocytes		Monocytes		Basophils		Eosinophils	
	Mean	s.d.**	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.
Low	2110	192	1494	322	10	6	82	39	10	10	57	21
High	10411	2003	4498	1802	527	258	297	160	79	27	739	301
Group	4641	1650	2491	1194	301	174	129	95	46	38	441	351

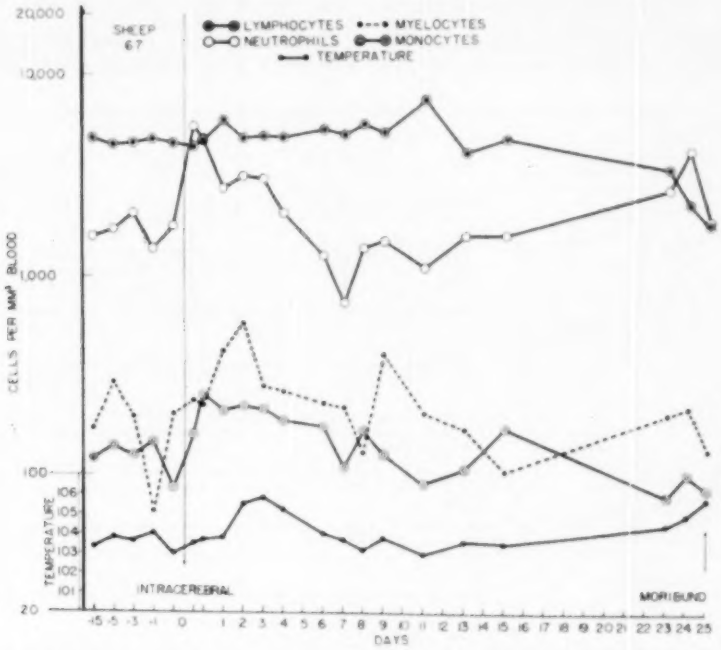
*259 observations on 13 sheep. **s.d. = standard deviation.

tures were slightly elevated on the day of inoculation and steadily increased to the time of death.

Sheep 67 provided an unusual reaction. There was a moderate increase of neutrophils after exposure and a drop to less than normal at seven days. On the twenty-third day, it appeared ill and counts of the blood showed a slight decrease of lymphocytes and increase of neutrophils. On the twenty-fourth day, the blood counts were similar to those of sheep with active infection (graph 5). The animal became moribund on the twenty-fifth day and was sacrificed. Attempts were made to isolate the organism from the brain, spleen, marrow, and lymph node with negative results. There was a localized accumulation of leucocytes in the arachnoid over the cerebral hemisphere, similar to that seen in other sheep with experimental infection.

Sheep 802 survived intracerebral exposure. The reaction of this sheep was characterized by a more marked and sustained increase of neutrophils. Blood counts returned to normal in six days. Temperature was elevated 3 to 3.5 F. for about three days.

Intravenous Exposure. — Seven sheep were used, 3 of which had from seven to ten previous exposures to *L. monocytogenes.* Two of the sheep had one previous exposure and 2 had no previous exposure. The reactions were different in these three groups of sheep.



Graph 5—Leucocyte counts and temperatures on sheep 67 after intracerebral inoculation of 0.25 ml. of *Listeria monocytogenes.* Counts of leucocytes are plotted on a logarithmic scale and temperature values follow arithmetic progression. On the day of exposure (0), counts were made three and five hours after inoculation. During the interval from six to sixteen days, the values may be considered normal.

Those with previous multiple exposures had a drop in number of neutrophils for the first three hours and a marked increase apparent at seven hours and lasting forty-eight hours. A normal level was reached in four to five days. Lymphocytes showed a drop about two to five hours after exposure, but had returned to normal in from one to four days. Marked elevation of temperature occurred (3 to 4 F.) in from three and one-half to four hours. Normal temperatures were obtained in two to three days. The record of sheep 286 serves as

an example of reaction obtained (graph 1).

Two sheep which had only one previous exposure showed a marked increase in neutrophils following a preliminary slight decrease. The peak existed at seven to twenty-four hours and the normal levels were reached at three days. Lymphocytes decreased at from one to seven hours and returned to their normal level at twenty-four hours in 1 sheep and at three days in the other sheep. Temperatures increased 2.5 to 3.5 F. at four to seven hours and were normal at twenty-four hours. The 2 sheep without previous exposure had an increase of neutrophils at seven hours without a preliminary decrease. The lymphocytes of both sheep were moderately depressed on the inoculation day. Both neutrophils and lymphocytes were at normal levels in 1 sheep at forty-eight hours. In the other sheep, lymphocyte numbers were normal at twenty-four hours and neutrophils were normal at seventy-two hours. The temperature peak was 4.5 to 5 F. above normal and the temperature remained elevated through the fifth day.

Monocytes varied through what was considered a wide range in all sheep given

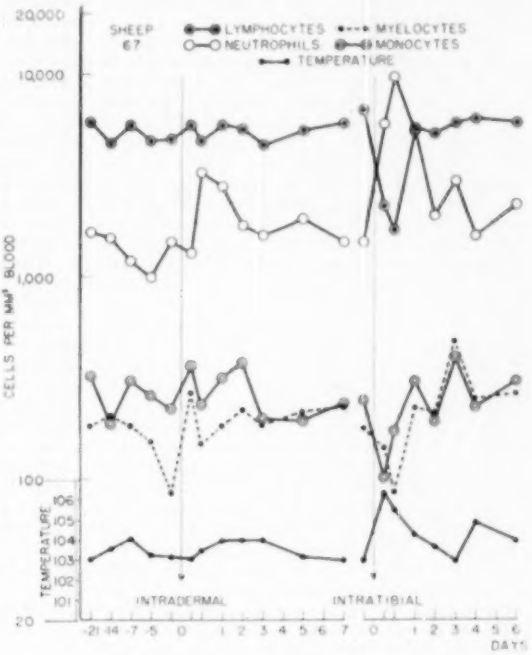
TABLE 2—General Summary of Reactions of Sheep to Experimental Listeriosis

Method of exposure	No. trials	Previous uses	Died (%)	Blood cell reactions		Temperature increase
				Neutrophils	Lymphocytes	
Carotid	5	None	100	Increased, marked 1st 2 days, slight to death	Decreased, slight 1st day, variable to death	Highest 2nd day, variations to death
	8	Once	38	Increased, marked 1st 2 days, variable 3 to 29 days	Decreased, slight 1st day, persists 1 to 4 days	Variable 1st day, higher 2nd day, persists 2 to 7 days
	5	Multiple	0	Increased, marked 1st 2 days, slight 2 to 7 days	Decreased, slight 1st day, persists 1 to 4 days	Higher 1st day, persists 1 to 2 days
Cerebral	1	None	100	Increased, gradual to extreme on 2nd day	Decreased, slight 1st day, variable to death	Slight 1st 2 days, extreme 3rd day at death
	7	Multiple	57	Increased, gradual to marked 2nd day, persists to death	Decreased, slight 1st day, slight to death	Variable, persists to death
Intravenous	2	None	0	Increased, moderate 1st 2 days, slight 1 to 5 days	Decreased, moderate 1st day, normal 2nd day	Highest 1st and 2nd day, persists 5 days
	2	Once	0	Decreased, slight at 10 min. Increased, extreme 7 to 24 hr., slight to 3rd day	Decreased, marked 1st day, slight 2nd day; normal 3rd day	High 1st day, persists 2 days
	3	Multiple	0	Decreased, marked at 3 hr.; Increased, marked at 1 day, persists 4 to 7 days	Decreased, marked 1st day, slight 2nd day; persists 1 to 5 days	Moderate 1st day, persists 1 to 3 days
Subcutaneous	4	None	0	Increased, moderate 1st 2 days, persists 3 to 5 days	Decreased, normal at 2nd or 3rd day	No change
	1	Once	0	Increased, marked 1st 2 days, persists 3 to 5 days	No change	Slight for 3 days
	6	Multiple	0	Increased, marked 1st 2 days, persists 3 to 5 days	No change	Slight for 2 to 5 days
Median artery	2	None	0	Increased, marked 1st 2 days, later normal	Decreased, moderate 1st day only	High 2nd to 7th day
Dermal	5	Multiple	0	Increased, moderate at 24 hr., later normal	No change	No change
Tibial	5	Multiple	0	Increased, moderate 1st and 2nd day, normal after 2nd day	Decreased, moderate 1st day, persists to 3rd day	Higher 1st day, persists 1 to 3 days
Muscular	4	Multiple	0	Increased, moderate 1st day, marked 2nd day, normal 4th day	Decreased, moderate 1st day, persists 1 to 3 days	Higher 1st day, persists 2 days
Peritoneal	3	Multiple	0	Increased, moderate 1st day, marked 2nd day, persists 4 to 5 days	No change	No change

cultures of *L. monocytogenes* via jugular vein.

Subcutaneous Exposure. — Nine sheep were given cultures of *L. monocytogenes* in the subcutaneous tissues and 2 were given a second similar exposure. Of the 11 trials, four were made in sheep not previously used, one in a sheep with one previous exposure, and six in sheep that had from seven to 15 previous exposures. The reaction of blood cells was essentially similar in all 11 trials (graph 3). The increase of neutrophils was moderate at seven hours and marked at twenty-four hours.

Exposure into Median Artery. — Two sheep not previously used were given *L. monocytogenes* culture in the median artery at a dosage of 0.05 ml. per pound body weight. Both had an increase of neutrophils (more marked in 1) one and one-half to five hours later. This lasted for two days. Lymphocytes were moderately decreased on the day of inoculation and later normal. Temperature was elevated from the second to seventh day following exposure.



Graph 6—Leucocyte counts and temperature reactions of sheep 67 after intradermal and intratibial (marrow) inoculation of 0.25 ml. of broth culture of *Listeria monocytogenes*. The animal had been exposed twice previously.

Counts of leucocytes are plotted on a logarithmic scale and temperature values follow arithmetic progression. On the days of exposure (0), counts were made prior to and at three and seven hours after injection.

Neutrophils reached normal preëxposure levels at four to five days. In two trials, normal levels were reached in one and three days and in two other trials at six days. The number of lymphocytes was slightly, if at all, affected. Myelocyte counts varied over their normal range. Body temperature was not greatly affected. In most instances, either no change or a slight increase of 1 F. was noted in the group whose first exposure was via the subcutis. Seven trials on sheep previously exposed showed a moderate temperature elevation of 1 to 2.5 F. which developed during the day of inoculation and lasted twenty-four to forty-eight hours.

One sheep was killed twenty-six days after exposure and attempts to isolate *L. monocytogenes* from the brain and visceral organs were negative.

Intradermal Exposure.—Five sheep, all of which had been previously exposed, were given 0.25 ml. of broth culture of *L. monocytogenes*. There was no local systemic reaction following this exposure. The reaction of blood cells was very slight with only a transient increase in neutrophils. (Sheep 67, graph 6, is typical.) Temperature was very slightly, if at all, affected.

Intratibial Exposure.—Five sheep were

exposed to *L. monocytogenes* by injecting 0.25 ml. of broth culture into the marrow cavity of the tibia. The operations were done under local anesthesia and with reasonably aseptic conditions. One animal (sheep 67) had been exposed three times previously and showed the most marked reaction (graph 6). Neutrophils increased and lymphocytes decreased with maximum and minimum levels noted at seven hours. Temperature increased 3 F. on the day of inoculation. The return to normal of both blood cells and temperature was rapid and complete in forty-eight hours. Two sheep had ten and 2 had 13 previous exposures. These had a similar but milder reaction to that shown in graph 6.

Intramuscular Exposure. — Four sheep were given cultures of *L. monocytogenes* in the biceps femoris muscle. The dosage was 0.05 ml. per pound body weight. This was injected at one site. One sheep had seven previous exposures, 2 sheep had 11, and 1 had 21 previous exposures. Three of the sheep reacted in a similar way. Neutrophils increased, reaching peak levels at thirty hours, and slowly returned to normal on the fourth day. Lymphocytes remained at preëxposure levels in 2 sheep. Lymphocytes were diminished slightly in 1 sheep and moderately in the other. The slight lymphopenia lasted three days in 1 sheep. Body temperature was irregular with 1.5 to 2.5 F. elevation at twenty-four and thirty hours. Normal temperature was found at forty-eight hours and remained so.

Per Os Exposure. — Three sheep were given *L. monocytogenes* cultures in amounts of 0.1 ml. per pound body weight. This was given in gelatin capsules. The sheep had from two to 18 previous exposures. The reactions of all 3 sheep were essentially the same. The increase of neutrophils was moderate and the highest point was noted at twenty-four to thirty hours. The increase persisted four to five days. Lymphocyte numbers and body temperature remained within the normal range of each sheep.

DISCUSSION

Considerable variation was observed in the way sheep react to exposure with *L. monocytogenes* (table 2). Two principal factors seemed involved in determining the type of reaction, namely, the method of exposure and whether the sheep had been previously exposed to the organism.

Animals exposed by way of the carotid artery had a similar reaction of neutrophils, lymphocytes, and body temperature, irrespective of whether they had been previously exposed. The factor of previous exposure seemed to determine whether the sheep would die as a result of the intracarotid injection of *L. monocytogenes*. Injection of the culture into the median artery of sheep not previously used did not cause death. Since such susceptible sheep would die if exposed via carotid artery, direct transit to the brain must be one essential factor in the production of fatal listeriosis of sheep. Injection of a culture against the flow of blood in a ligated carotid artery probably reaches the brain through anastomosis with the vertebral artery. A similar type of reaction of blood cells and body temperature followed intracerebral exposure with *L. monocytogenes*.

Intravenous exposure yielded different results. None of the sheep died after receiving the organism in the jugular vein. Sheep which had received one or more previous exposures to *L. monocytogenes* had a preliminary decrease in neutrophils immediately after injection which was followed by the characteristic increase of neutrophils at a later time. This immediate decrease of neutrophils was more marked in those sheep which had several previous exposures to *L. monocytogenes*.*

The other methods of exposure, subcutaneous, intradermal, intratibial, intramuscular, and *per os* caused an increase in neutrophils, the extent and duration of which was somewhat variable. Some differences were noted in the reaction of circulating lymphocytes. While in general the number of lymphocytes decreased after the introduction of *L. monocytogenes*, there was no change of lymphocytes in sheep injected intradermally or *per os*. The sheep receiving a subcutaneous exposure and which had a previous exposure to the organism showed no change of lymphocytes.

Usually, there was a febrile response following introduction of *L. monocytogenes*. The extent of this varied with the different methods of injection. Subcutaneous exposure of fresh sheep and intradermal and *per os* exposure caused no change in body temperature.

It is difficult to compare the observations with the reported work of others, for, in many instances, relative percentage values for leucocytes were given without stating the total number of leucocytes in the circulating blood, and, in others, only general comments were made. Gill[13] reported observations on three spontaneous cases of listeriosis in which he observed a high number of neutrophils and a low number of lympho-

*The decrease of neutrophils was not necessarily related to the larger amount of culture given intravenously, for, in another experiment where 2 sheep were used, the dose was reduced by one half and a similar response was obtained.

cytes. These were based on differential counts only and expressed as percentage values. Gill[14] also made differential counts on experimentally produced cases of listeriosis in sheep. One sheep, exposed by way of the carotid artery, had 61 per cent neutrophils in the blood at time of death. In 1 sheep exposed by way of the nose, there was 67 per cent neutrophils in the blood at time of death. Graham, Dunlap, and Brandly[15] reported that the blood picture of 5 field cases of listeriosis in sheep showed no deviation from normal. Graham, Dunlap, and Levine[16] reported observations on the blood of experimental listeriosis in sheep. Four animals were injected in four different ways. The lamb exposed intracerebrally died in two days and showed a marked increase of neutrophils at one day. At this time, the lymphocytes were decreased and the monocytes had increased. The sheep injected intravenously died in eighteen days. There was a relative percentage increase of neutrophils and decrease of lymphocytes at twelve days. Only a slight percentage decrease of neutrophils was observed in a sheep exposed on the conjunctiva. A lamb exposed by feeding culture through a stomach tube showed a relative decrease of neutrophils and an increase of lymphocytes at ten days. Blood counts were also reported on 3 field cases in which there was a relative increase of neutrophils and a decrease of lymphocytes in 2 of them; the third case was normal.[17] Graham, Morrill, and Levine[19] reported a general decrease in the total leucocyte count and a relative increase of neutrophils following the intravenous injection of culture into sheep which had previously received a killed bacterin of *L. monocytogenes*. Jungherr[22] reported a normal differential count of 1 spontaneous field case of listeriosis and 1 experimentally produced case following exposure via the carotid artery. Muth and Morrill[24] reported a differential count on a spontaneously infected sheep in which they indicated neutrophils at 44 per cent, lymphocytes at 47 per cent, and monocytes at 5 per cent. Biester and Schwarte[2, 3] reported a definite increase of monocytes in field cases of listeriosis in both sheep and swine. The apparent contradictions of reports in the literature are no doubt due to the fact that relatively few observations had been made. Furthermore, these observations were reported in terms of percentage values rather than absolute values for the different types of leucocytes.

It should be noted that Julianelle[21] observed variations in the blood reaction of rabbits to *L. monocytogenes*. Sometimes, there was neutrophilic leucocytosis rather than monocytosis and even a leucopenia rather than leucocytosis.

It would seem that *L. monocytogenes* has the property to stimulate a monocytosis in rabbits[23, 1, 21] and chickens.[5] Guinea pigs are reported to respond with a monocytosis.[19, 20] White mice are reported to show a monocytosis,[20] or to show a relative increase of neutrophils when exposed to strains of *L. monocytogenes* coming from chickens.[9] Chapman[7] has reported a 25 per cent monocyte value with a leucocyte count of 16,400 in a spontaneous case of the dog. The blood cell reaction in human beings infected with *L. monocytogenes* is still not clear.[20] Burn[5] and Carey[6] have reported an increase of neutrophils in human cases.

Sufficient data have been accumulated in our study to justify the comment that *L. monocytogenes* is capable of producing a neutrophilic leucocytosis and a suppression of the number of lymphocytes in the circulating blood of sheep. The rapidity and intensity of increase in number of neutrophils is indeed surprising. Conway[10, 11] has shown the rapidity with which pathologic changes can occur in experimental listeriosis of the rabbit. As previously mentioned, the reaction of neutrophils and lymphocytes as well as the temperature reactions are controlled by two factors. One is the method of exposure to the organism and the other is probably related to the immunity status of the individual sheep. Evidence of such immunity is rather strikingly shown in the group of sheep receiving *L. monocytogenes* via the carotid artery. One hundred per cent of those sheep not previously exposed died, while, of sheep which had one previous exposure, only 38 per cent died, and, of sheep receiving multiple previous exposures, none died following intracarotid exposure. This resistance is perhaps associated with the so-called "blood-brain barrier" to infection. The reactions of the blood cells and temperature could not be associated with the immunity status of the sheep. The reactions of the blood were, however, indicative of the outcome following experimental inoculation. For, when the outcome was fatal, the characteristic changes either persisted in the blood or became manifest in the terminal stages following a period of apparently normal levels.

The finding of immature neutrophils in the circulating blood of apparently normal sheep was unexpected. No detailed studies on this aspect have been found in the available literature. The correctness of the observation is substantiated by an increase of such cells following a heavy demand for

neutrophils, as well as by tracing the morphologic development through different stages (plate 1). In some instances, the large number of immature granulocytes, some of which could be traced back to the progranulocyte stage, created the appearance of a "leucemoid" reaction.

SUMMARY

The blood cell and body temperature reactions of 28 sheep were studied in 63 exposures of the sheep to *Listeria monocytogenes*. The exposures were made by different routes, including the carotid artery, the cerebrum, the jugular vein, the subcutaneous tissue, the dermis, the bone marrow of the tibia, muscle tissue, and *per os*. In general, there was an immediate increase of neutrophils and a decrease of lymphocytes. Body temperature was in general increased. The degree of change was related to the method of exposure and perhaps the immunity status of the sheep. Fatal infections were induced by exposure into the carotid artery or directly into the cerebrum only. Sheep which had been previously exposed to the organism were relatively resistant to exposure by way of the carotid artery, so far as a fatal outcome is concerned.

Immature neutrophils are present in the circulating blood of apparently normal sheep. Following a heavy demand for neutrophils, as a result of exposure to *L. monocytogenes*, many immature neutrophils were found in the blood.

References

[1]Barber, M.: A Comparative Study of Listerella and Erysipelothrix. J. Path. and Bact., *46*, (1939): 11-23.

[2]Biester, H. E., and Schwarte, L. H.: Studies on Listerella Infection in Sheep. J. Infect. Dis., *65*, (1939): 135-144.

[3]Biester, H. E., and Schwarte, L. H.: Listerella Infection in Swine. J.A.V.M.A., *96*, (1940): 339-342.

[4]Biester, H. E., and Schwarte, L. H.: Listerellosis in Swine and Criteria for Diagnosis. Rep. Forty-fourth Ann. Meeting, U. S. Livestock Sanitary A., (1940): 42-47.

[5]Burn, C. G.: Clinical and Pathological Features of an Infection Caused by a New Pathogen of the Genus Listerella. Am. J. Path., *12*, (1936): 341-348.

[6]Carey, B. W., Jr.: Infection with an Organism of the Genus Listerella. Report of a Case of Acute Cerebrospinal Meningitis with Recovery. Jour. Pediat., *8*, (1936): 626-629.

[7]Chapman, M. P.: Listerellosis in a Dog. A Field Case. North Am. Vet., *28*, (1947): 532-538.

[8]Cole, C. R.: Listerellosis in a Hereford Cow. J.A.V.M.A., *109*, (1946): 216-217.

[9]Cole, R. K.: Listeria (Listerella) Infection in a Fowl. Poult. Sci., *20*, (1941): 28-32.

[10]Conway, E. A.: Reaction of Lymphatic Tissue in Early Stages of *Bacterium Monocytogenes* Infection. Arch. Path., *25*, (1938): 209-227.

[11]Conway, E. A.: Reaction of Lymphatic Tissue of Rabbits to Repeated Injections of *Bacterium Monocytogenes*. J. Infect. Dis., *64*, (1939): 217-240.

[12]Gifford, R., and Jungherr, E.: Listeriosis in Connecticut with Particular Reference to a Septicemic Case in a Wild Raccoon. Cornell Vet., *37*, (1947): 39-48.

[13]Gill, D. A.: "Circling Disease" of Sheep in New Zealand. Vet. J., *87*, (1931): 60-74.

[14]Gill, D. A.: Circling Disease: A Meningo-Encephalitis of Sheep in New Zealand. Notes on a New Species of Pathogenic Organism. Vet. J., *89*, (1933): 258-270.

[15]Graham, R., Dunlap, G. L., and Brandly, C. A.: Ovine and Bovine Listerellosis in Illinois. Science, *88*, (1938): 171-172.

[16]Graham, R., Dunlap, G. L., and Levine, N. D.: Studies on Listerella. III. Experimental Listerellosis in Domestic Animals. Cornell Vet., *30*, (1940): 268-290.

[17]Graham, R., Hester, H. R., and Levine, N. D.: Studies on Listerella. II. Field Outbreaks of Listerellosis in Sheep and Cattle. Cornell Vet., *30*, (1940): 97-111.

[18]Graham, R., Levine, N. D., and Morrill, C. C.: Listerellosis in Domestic Animals. Illinois Agric. Exper. Sta., Bull. 499, 1943.

[19]Graham, R., Morrill, C. C., and Levine, N. D.: Studies on Listerella IV. Unsuccessful Attempts at Immunization with Living and Dead Listerella Cultures. Cornell Vet., *30*, (1940): 291-298.

[20]Julianelle, L. A.: The Function of Listerella in Infection. Ann. Int. Med., *14*, (1940): 608-620.

[21]Julianelle, L. A.: Biological and Immunological Studies of Listerella. J. Bact., *42*, (1941): 367-383.

[22]Jungherr, E.: Ovine Encephalomyelitis Associated with Listerella Infection. J.A.V.M.A., *91*, (1937): 73-87.

[23]Murray, E. G. D., Webb, R. A., and Swann, M. B. R.: A Disease of Rabbits Characterized by a Large Mononuclear Leukocytosis Caused by a Hitherto Undescribed Bacillus: *Bacterium Monocytogenes*. J. Path. and Bact., *29*, (1926): 407-439.

[24]Muth, O. H., and Morrill, D. R.: An Outbreak of Listerellosis in Oregon Sheep. J.A.V.M.A., *100*, (1942): 242-243.

[25]Nyfeldt, A.: Klinische und experimentelle Untersuchungen über die Mononucleosis Infectiosa. Folia haemat., *47*, (1932): 1-144.

[26]Osgood, E. E. (Chairman): First Report of the Committee for Clarification of the Nomenclature of Cells and Diseases of the Blood and Blood Forming Organs. Am. J. Clin. Path., *18*, (1948): 443-450.

[27]Prescott, J. R., Keener, H. A., Dobrovolny, C. G., and Percival, G. P.: The Normal Blood Picture of Sheep. Anat. Rec., *99*, (1947): 7.

[28]Seastone, C. V.: Pathogenic Organisms of the Genus Listerella. J. Exptl. Med., *62*, (1935): 203-212.

The Neuropathologic Diagnosis of Hog Cholera

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HOG CHOLERA presents many differential diagnostic problems. This paper deals with the authors' experiences in the neuropathologic diagnosis of hog cholera. Boynton *et al.*[3] pointed out that the more experienced the clinician the more difficulty he has in making a diagnosis, owing to his knowledge of overlapping symptoms and lesions. At any rate, hog cholera may be confused with other diseases.

Manninger,[27] Pinkerton,[29] and Seghetti[30] reported cases of salmonellosis which produced symptoms and lesions of hog cholera. Udall[42] stated that hog cholera might be confused with erysipelas and pasteurellosis. Hutyra *et al.*[16] listed erysipelas, salmonellosis, Aujeszky's disease, and swine influenza as confusing entities. Kernkamp and Fenstermacher[21] pointed out that the disease is difficult to diagnose when it exists in the subtypical form; they further remarked that most people are reluctant to accept a diagnosis of hog cholera when the animals had already been vaccinated.

In Connecticut, the majority of swine herds are garbage fed, and as a result are exposed to various porcine infections. Hog-cholera vaccination, together with hemorrhagic septicemia vaccination, is practiced widely as the work is done at state expense.

Transmission of the disease to susceptible animals is the diagnostic method of choice, but is usually impractical owing to a lack of susceptible stock. To circumvent this disadvantage, a more rapid and economical method is needed.

King and Wilson,[22] as well as Lewis and Shope,[25] found leucopenia in the early stages of hog cholera to be of diagnostic assistance. Boynton *et al.*[3] observed intranuclear "inclusion bodies" in the epithelium of the gall bladder, which these authors considered specific. European workers examined the brain histologically as it is known that hog cholera causes symptoms referable to the central nervous system. Fekete[7] reported rabies-like symptoms, Eberdeck,[6] weakness and posterior paralysis, Udall,[42] motor irritation as manifested by twitching and convulsions, and Hutyra *et al.*,[16] depression, convulsions, weakness, and vomiting.

REVIEW OF LITERATURE

Huguenin[14] noted hemorrhages, edema, hyperemia, and inflammatory processes in 20 per cent of the brains examined.

Brunschwiler[4] reported dilated blood vessels, lymphocytic perivascular infiltration, and endothelial swelling in 39.3 per cent of the brains examined.

Röhrer[33] found microscopic changes of the brain in 75 per cent of his cases. The most conspicuous changes consisted of vascular infiltrations by histiocytes. He also observed neuronophagia and both intranuclear and intracytoplasmic inclusion bodies in the neurons. Röhrer and Graf[35] considered hog cholera as a true encephalitis to which the latter author[11] added the involvement of the cord (myelitis).

Laumen[18] regarded vascular and perivascular infiltrates by lymphocytes to be the significant lesion. He found them to be common in the choroid plexuses, cerebral peduncles, corpus striatum, thalamus, Ammon's horn, cerebellar pia, and cerebral cortex.

Seifried[39] saw neuropathologic alterations in 33 of 39 artificially induced cases of hog cholera. He interpreted the process as a true encephalitis, with or without myelitis, which was characterized by vascular and perivascular infiltrations by lymphocytes, mononuclears, and some plasma cells. Focal glial proliferations were observed in the vicinity of blood vessels. Satellitosis and neuronophagia were infrequent. The lesions were seen as early as six days postinfection. In subsequent communications,[40] he pointed to the periventricular areas and the ventral portions of the brain as the preferential sites of the encephalitic process; and in 1934,[41] classified hog cholera as encephalomyelitis type II with approximately equal changes in the gray and white matter, while encephalomyelitis type I showed changes primarily in the gray matter.

Salvalaglio[36] saw endothelial proliferation and lymphocytic perivascular infiltration in the brains of 29 of 35 hogs dead of hog cholera.

MATERIALS AND METHODS

The material examined was obtained from hogs submitted to this laboratory for autopsy. The earliest specimen was submitted Jan. 8,

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1938, and the remainder up to June, 1949. If, as Lüttschwager[20] believed, the character of the virus may vary from year to year, the eleven-year period should cover at least some of these variations. In all, 89 cases of diseased hogs were examined and 35 normal hogs were secured from a nearby abattoir and from an experimental vitamin A project.

Bateriologic examinations were made of the viscera; rabbit inoculations were used to rule out Aujeszky's disease. Tissue blocks were taken of the viscera and the visibly affected lymph nodes for histopathologic study.

The brains were fixed in 10 per cent formol-saline. The majority of the brains were cut crosswise at the levels of the optic chiasma, mammillary body, oculomotor nerve, pons, and the medulla. Representative blocks were embedded in paraffin and stained with hematoxylin-triosin.

RESULTS

The anatomic alterations observed in the porcine brain could be divided roughly into those involving the ectodermal and those involving the mesodermal elements. The former changes were seen primarily in young swine without any relation to antecedent diseases and were considered nonspecific. The latter ones were representative of a nonsuppurative encephalitis, which, in this region, was considered to be due to the virus of hog cholera. The data are summarized in table 1.

Nonspecific Ectodermal Gliosis.—Cellular aggregates were frequently found in closely knit groups as subependymal nests (fig. 1) or as more loosely formed foci periventricular to the lateral ventricles (fig. 2). In hematoxylin-triosin preparation, the nuclei appeared naked and were smaller than the nuclei of astrocytes, oval to round, and hyperchromatic. In frozen sections stained with silver carbonate (McClung[28]) these glial cells showed few stubby processes characteristic of oligodendroglia (fig. 3). Similar foci were accorded pathologic significance by Röhrer[33] and Eberdeck.[6] On the other hand, Laumen,[24] Röhrer and Graf,[35] and Köbe and Schmidt[23] stated that they occurred in normal swine and were nonpathologic in character. In our study of 35 normal swine, such glial foci were seen in 24 (table 1); among 89 swine with various noninfectious and infectious diseases, they appeared in 37 cases. There was no obvious relation of ectodermal glial foci to vaccination, but there did seem to be a trend toward their decrease in incidence and intensity with advancing age.

The functional roles of neuroglia and of oligodendroglia in particular are not definitely understood. According to Freeman,[9] the latter may be actually responsible for myelinogenesis, while Ranson[30] ascribes to them chiefly a regulatory and metabolic function. If this concept were correct, the increased occurrence of such cellular foci could be interpreted as aberrant oligodendroglia elements in the developing brain. Although these cellular aggregates do not belong to the normal cytoarchitecture of the porcine brain, they occur with such regularity and without relation to recognizable pathologic processes that they must be considered within the range of normal histoanatomic variations and should not be accorded pathologic significance.

Specific Mesodermal Reactions.—The lesions associated with hog cholera were found in the following order of frequency: vascular and perivascular cuffs, microgliosis, leptomeningeal infiltrates, capillary hemorrhages, and hyalinization of the vascular wall.

The vascular and perivascular infiltrates varied from a few cells (fig. 5 to 7) in the Virchow-Robin space to lesions crowding the perivascular space of His (fig. 10, 12). The cellular elements appeared to be chiefly lymphocytes with an occasional plasma cell. The lymphocytes were morphologically identical with similar cells seen in other sections of the same animal and corresponded to the description of the cells involved in nonsuppurative encephalitides of man.[32]

The vascular and perivascular lesions seen in hog cholera were generally well pronounced. Of 31 cases not complicated with other conditions, only 3 failed to exhibit them. Of 13 cases of hog cholera complicated with secondary conditions such as salmonellosis, only 1 failed to show them.

In comparison with the vascular changes, the microglial foci occurred much less frequently. These aggregates were of generally loose composition and irregular occurrence in either the white or the gray matter. The nuclear components were small, polygonal to oblong, strongly hyperchromatic, and readily identifiable as microglia or scavanger cells. They differed by virtue of their distinct morphology and variable localization from the nonspecific oligodendroglia aggregates described above.

Leptomeningeal infiltrates (fig. 8, 9) were not common lesions. Of all hog-cholera cases, only 4 showed a recognizable meningitis, 4 others were considered questionable. The cellular elements involved were obviously lymphocytes.

Capillary hemorrhages (fig. 10) were relatively infrequent. Their presence was expected since the virus of hog cholera is regarded as endotheliotropic by Eberdeck.[6]

Further evidence for this property is furnished by the well-known visceral hemorrhages which have been tabulated in detail by Kernkamp.[20]

Hyaline degeneration (fig. 11) of the vascular walls was likewise an infrequent lesion. The hyalin was of the connective tissue type and was seen primarily in the small arteries, involving the media. The process might be considered an extension of the endotheliotropic properties of the virus. Hutyra *et al.*[16] reported hyalinization of the blood vessels of the brain to the point of bringing about occlusion of the lumens.

The localization of the specific mesodermal lesions was not confined to any particular anatomic portion of the brain, although there was relative predilection for the brain stem (table 1). The gray and white matter seemed to be equally involved in the pathologic process. Although no total count of the lesions was made, a random sample of 166 sections showed specific changes in the white matter of all sections, and in the gray matter in 134 sections,—an insignificant difference probably conditioned by the relative anatomic distribution of these two substances.

The intensity of the pathologic alterations was not paralleled by the reported severity and duration of the disease. For example, an adult sow which presented barely detectable signs and symptoms, and

TABLE 1—Incidence of Neuroanatomic Alterations in Various Porcine Conditions

	Diagnosis		Incidence of brain changes			Number of cases			Total incidence of changes
	Primary	Secondary	Cerebrum	Cerebellum	Brain stem	Vacc.	Non-vacc.	Total	
Noninfectious diseases	Anemia		3		IIsl	2	6	8	3, IIsl
	Deficiencies (B complex)		2			2	1	2	2
	Deficiencies (unidentified)		1			2	3	5	1
	Hypoglycemia		1		Isl		2	2	1 Isl
	Normal <6 days old		12				14	14	12
	Normal <30 days old		6				6	6	6
	Normal approx. 200 days old		1			4		4	1
	Normal approx. 260 days old		4			11		11	4
	Poisoning—salt						1	1	
	Poisoning—veratrin					1		1	
	Undiagnosed		2			2	1	3	2
Infectious diseases	Balantidiosis					1		1	1
	Cholera		10, 10*, XXII	6*, XX	10*, XXVIII	19	12	31	10, 10*, XXVIII
	Cholera	Coccosis	1, I			1		1	1, I
	Cholera	Lead poisoning	1		I		1	1	1, I
	Cholera	Pasteurellosis	2, 1*, II	1*, II	2*, II	2		2	2, 2*, II
	Cholera	Salmonellosis	2, 3*, IV	5*, III	2*, VII	8		8	2, 5*, VII
	Cholera	Sulfa intoxication	1		I	1		1	I
	Coccosis		1				1	1	1
	Erysipelas		1				2	2	1
	Hemorrhagic dysentery					1		1	
	Lymphangitis					1		1	
	Moniliasis		1				1	1	1
	Omphalitis		1				3	3	1
	Pasteurellosis		3			2	1	3	3
	Pneumonia		2				2	2	2
	Salmonellosis		2			3		5	2
	Tetanus						1	1	
	Tuberculosis				Isl	1		1	Isl

Arabic numerals indicate number of cases showing nonspecific (ectodermal) oligodendroglia foci.
Arabic numerals with asterisk indicate number of cases showing specific (mesodermal) microglial foci.
Roman numerals indicate number of cases showing specific (mesodermal) vascular and perivascular infiltrates.
Roman numerals with sl indicate number of cases showing very slight vascular infiltration.
Blank space indicates no findings.

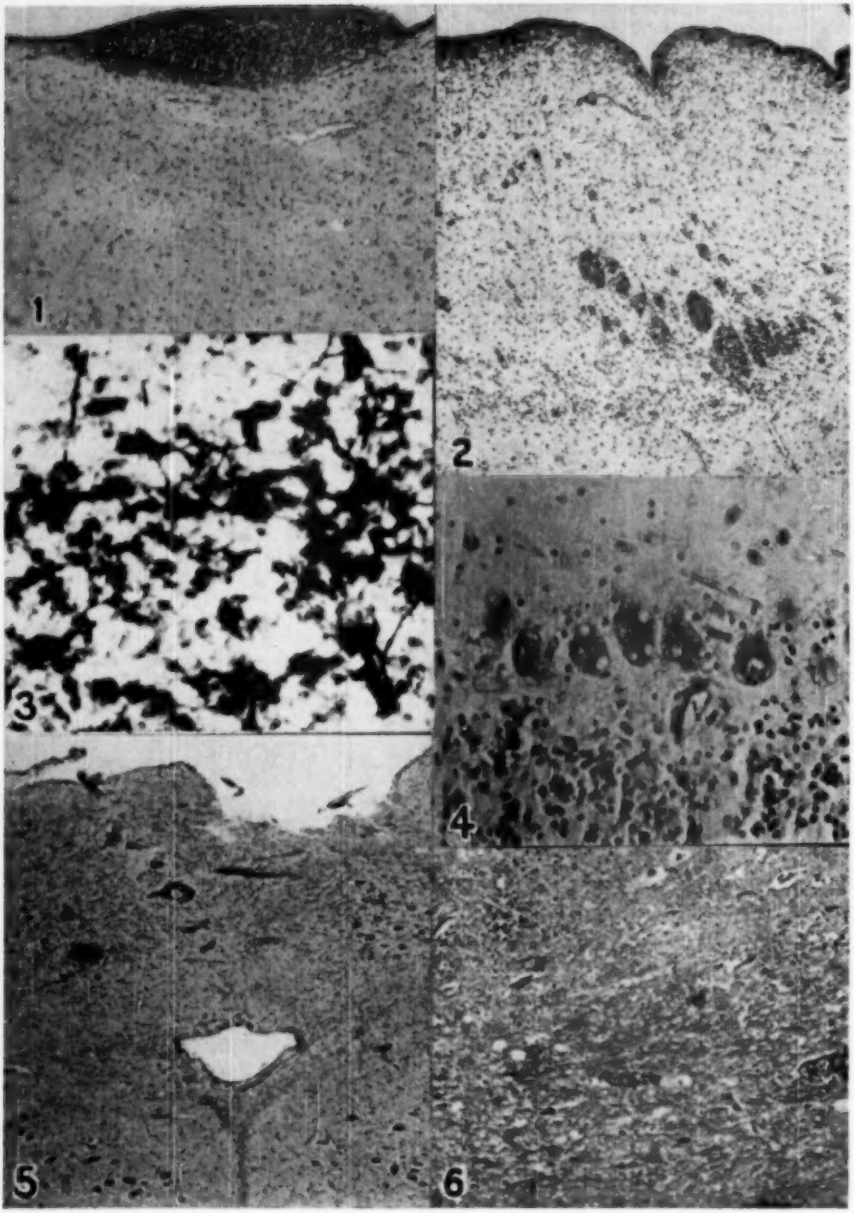

PLATE I

yielded hog-cholera virus on inoculation, manifested diffuse and marked encephalitic changes. These observations corroborated the findings of Laumen[24] on spontaneous cases and those of Seifried[39] on induced cases of hog cholera. A similar situation has been reported for 24 cases of anterior poliomyelitis in man by Bodian.[2]

Vaccination with serum and virus before the onset of the disease was apparently not capable of inducing neuropathologic lesions. According to the data presented of 35 vaccinated and 45 nonvaccinated cases, not presenting evidence of hog cholera, none showed lesions herein considered specific.

Mesodermal Reactions in Other Diseases.—Mesodermal reactions are seen in at least four other diseases of swine: Aujeszky's disease, Teschen disease, rabies, and listeriosis.

Aujesky's disease, according to Hurst,[15] causes a nonsuppurative encephalitis with meningitis, perivascular infiltration, and microglial proliferation in the gray and white matter. Hirt[13] saw essentially the same lesions, an opinion concurred in by Joest.[17] Salyi[37] considered the brain lesions as identical with those of hog cholera. Shahan *et al.*[42] also saw meningitis, microglial proliferation, and perivascular infiltration as noted previously. Ray[31] and Shahan *et al.*[42] pointed out that it is possible to differentiate hog cholera from Aujeszky's disease on the basis of animal susceptibility, since rabbits and mice readily develop the latter disease. Aujeszky's disease has not been seen in Connecticut so that it is not considered to be of differential diagnostic importance.

Teschen disease or infectious porcine encephalomyelitis has thus far been reported only from Central Europe. Diernhofer[5] described its neuropathology as chiefly occurring in the gray matter and the ventral parts of the spinal cord, and as consisting of neuronal destruction, perivascular infiltration by lymphocytes, meningitis, and gliosis. Sometimes the condition represented a definite anterior poliomyelitis, a feature confirmed by Kaplan and Meranze.[19] Fortner[8] could produce the disease by intranasal injection of susceptible swine, but not of other animals; he saw vascular and perivascular infiltrates, and gliosis in the brain and cord. Grau[12] observed perivascular infiltration, diffuse and focal gliosis, neuronal degeneration, meningitis, and filling of the central canal of the spinal cord with lymphocytes. Kaplan and Meranze[19] felt that Teschen disease could be differentiated from Aujeszky's disease by symptoms and by animal inoculation, and from hog cholera by symptoms.

Rabies is known to cause a nonsuppurative encephalomyelitis which can attack all parts of the brain and cord, but may be most pronounced in the central nervous organs functionally related to the peripheral sites of infection.[17] Although the lesions have the usual characteristics of mesodermal reactions, they may occur selectively in the ganglion nodosum of the vagus nerve, and be accompanied by the appearance of specific Negri bodies. The disease has not been re-

Legends for Figures on Opposite Page

Description of Figures

All figures are photomicrographs of hog-brain sections embedded in paraffin and stained with hematoxylin-triosin, except figure 3, which is a frozen section, stained with Penfield's second modificaton of Hortega's silver carbonate method for oligodendroglia.

Fig. 1—One-day old. Normal. Ammon's horn shows large subependymal oligodendroglia focus projecting into lateral ventricle. x 70.

Fig. 2—One-day old. Normal. Same specimen as figure 1. Corpus callosum shows several periventricular oligodendroglia foci in low center. Lateral ventricle at upper surface shows slight diffuse subependymal cell aggregates. x 70.

Fig. 3—Seven-days old. Normal. Corpus callosum shows a periventricular focus similar to that in figure 2. Special stain reveals characteristic short processes of oligodendroglia. x 500.

Fig. 4—Approximately 105-days old. Vitamin B-complex deficiency. Vaccinated forty-two days previously. No gross lesions. Cerebellum shows marked vacuolation of Purkinje cells. x 200.

Fig. 5—One hundred and twenty-days old. Salmonellosis bacteriologically and hog cholera histologically. Vaccinated sixty days previously; treated with serum and mixed bacterin ten days previously. Gross pneumonia. Posterior aspect of corpora quadrigemina. Several perivascular cuffs dorsal to the third ventricle are diagnostic of hog cholera. x 27.

Fig. 6—One hundred and eighty-days old. Pasteurellosis bacteriologically and hog cholera histologically. Vaccinated one hundred and twenty days previously. Grossly "turkey egg" kidney and pneumonia. Thalamus shows two perivascular infiltrates in lower half and three microglial foci in upper half. x 90. Note difference in size between mesodermal glial cells in figures 6 and 11 and ectodermal glial cells in figures 1 and 2.

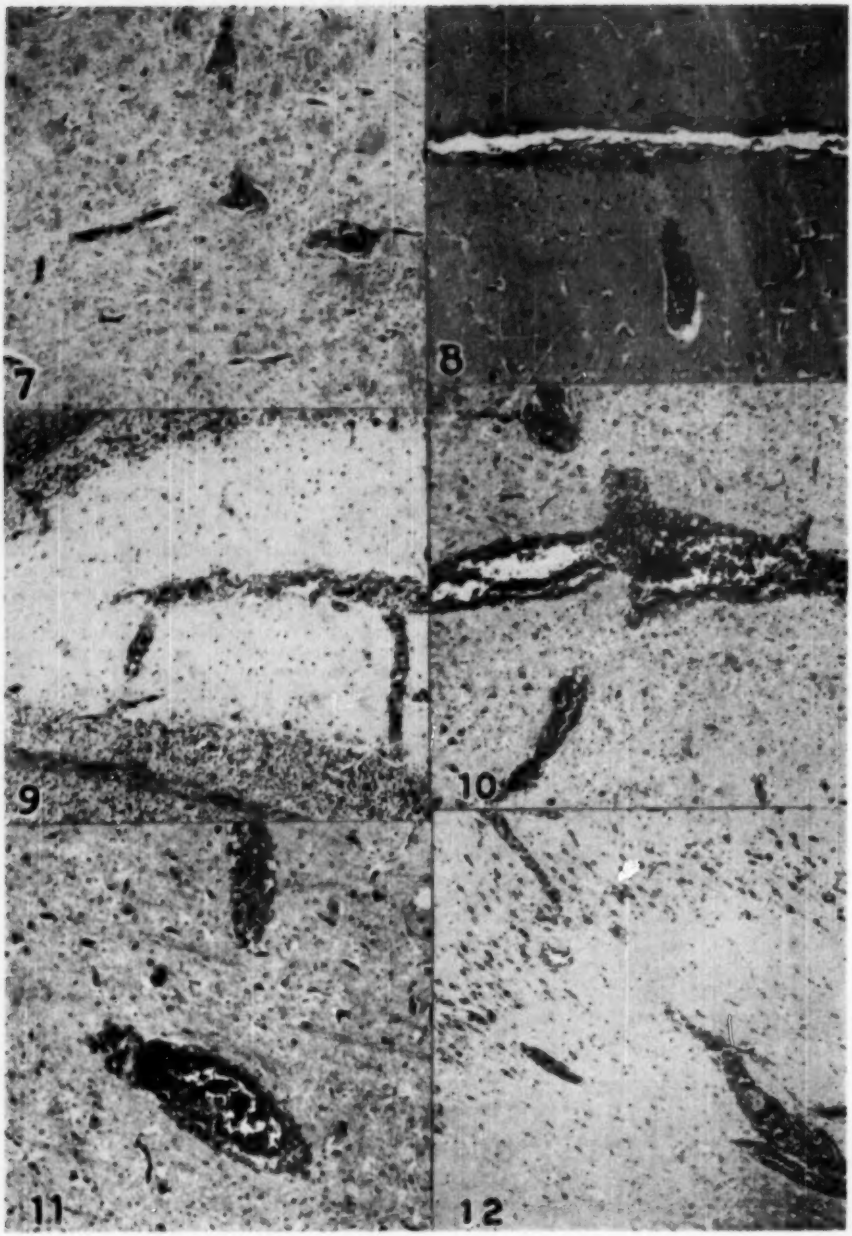

PLATE 2

ported as occurring in swine in Connecticut. It could be differentiated from hog cholera on the basis of symptomatology and animal inoculation.

Listeriosis has been reported in swine by Biester and Schwarte[1] and others. The disease causes marked mesodermal reactions of the meninges and the brain substance, and, in addition, necrotic foci and polymorphonuclear infiltrates. If the porcine lesions resemble those in ruminants as observed at this laboratory, they would be primarily confined to the brain stem. Furthermore, the disease can be diagnosed by bacteriologic methods and animal inoculation. Listeriosis in hogs is not known to occur in Connecticut according to a ten-year survey by Gifford and Jungherr.[10]

Neuropathology in Complicated Hog Cholera and Other Diseases.—Aside from 31 cases of uncomplicated hog cholera, which in most instances showed suggestive visceral lesions and were confirmed by neuropathologic examination, this study included 13 cases in which some other disease was demonstrated by postmortem and bacteriologic examination. The latter cases included pasteurellosis, salmonellosis, and sulfa and lead intoxication, each of which could be considered as an entity. Neuropathologic examination, however, revealed the presence of mesodermal lesions, which permitted interpretation of these cases as complicated hog cholera. Johannsen[18] saw many cases of complicated hog cholera and concluded that the encephalitic changes were not due to the secondary bacterial organisms.

Of 22 cases of various infectious diseases, none exhibited the marked mesodermal reactions seen in hog cholera. Seghetti[38] reported uncomplicated cases of salmonellosis, which largely presented the symptoms and gross lesions of hog cholera. Tissue filtrates failed to induce hog cholera; brain examinations were not reported. Salvalaglio[36] and Rohrer[34] both were emphatic as to the use of the brain for differential diagnosis.

The mesodermal reaction was conspicuously absent in all but 44 cases of hog cholera. Only 4 of the remaining 80 cases showed perivascular infiltrates and then so very slightly as to be easily overlooked. There was a marked difference in degree between the infiltrates seen in the 4 cases and the pronounced lesions in hog cholera.

Illustrative Cases

The following cases are reviewed to illustrate the application of neuropathology to the differential diagnosis of swine disease.

Case 1.—Of 150 garbage-fed hogs of various ages, most of which had been treated with crystal-violet vaccine eight months previous to the outbreak, 10 showed anorexia and depression during a two-week period and 3 died. Two veterinary consultants did not believe the condition to be hog cholera. A 200-lb sow, afebrile and reported as not eating, was sacrificed. Autopsy failed to reveal any significant gross changes. Neuropathologic examination, however, showed marked encephalitis. A 2-month-old susceptible pig, injected with citrated blood and ground spleen of this sow, showed high fever on the fifth day postinjection and a few typical renal hemorrhages, when sacrificed on the eighth day. The brain again showed pronounced encephalitis. In the field herd, a typical outbreak of hog cholera commenced about one week after the above mentioned episode and was brought under control by marketing all adult animals and simultaneous vaccination of the weanlings. This case could be interpreted as subtypical hog cholera, in the terminology of Kernkamp and Fenstermacher.[6]

Legends for Figures on Opposite Page

Description of Figures

All figures are photomicrographs of hog-brain sections embedded in paraffin and stained with hematoxylin-triosin.

Fig. 7—One hundred and eighty-days old. Same specimen as figure 6. Corpora quadrigemina show three perivascular infiltrates and several thickened capillaries due to endothelial hypertrophy. x 70.

Fig. 8—Same specimen as figure 6. Cerebrum shows one perivascular cuff in low center and subpial infiltrates in center. x 90.

Fig. 9—Same specimen as figure 6. Interfolia pia of cerebellum shows meningeal infiltration extending into Virchow-Robin spaces of two molecular layer vessels. x 70.

Fig. 10—Same specimen as figure 6. Posterior corpora quadrigemina show a large vein in center and a vessel above and below, with perivascular infiltrations. x 100.

Fig. 11—Same specimen as figure 6. Thalamus shows in low center an arteriole primarily with vascular infiltration; the vascular wall shows hyaline necrosis in upper aspect and hemorrhage in left pole. High center shows a typical perivascular focus. Upper left corner shows a microglial focus. x 70.

Fig. 12—Same specimen as figure 6. Cerebrum. Cortex shows small to large perivascular infiltrates. x 70.

Case 2.—Of 48 hogs, constituting the herd of a penal institution, which were fed garbage largely consisting of potato salad, all had been vaccinated under state supervision, but over two thirds had succumbed to an illness diagnosed by 2 veterinarians as hog cholera. Two live specimens showed convulsions; the 1 sacrificed failed to reveal gross lesions but showed marked vacuolation of the cerebellar Purkinje cells (fig. 4) and the large neurons of the Ammon's horn; the other was treated with vitamin B complex and recovered within six days. A similar response was obtained in the field. In this instance, the neuropathologic examination ruled out the possibility of hog cholera.

Case 3.—In a herd of 500 garbage-fed hogs, a group of 175, said to have been vaccinated twice, became ill and suffered high mortality. Two veterinarians believed the condition to be pasteurellosis, on the basis of gross pneumonic and bacteriologic findings. Treatment with hemorrhagic septicemia bacterin and sulfonamides was ineffective. Three carcasses and one head received as specimens showed pronounced encephalitis, thereby establishing a diagnosis of hog cholera in spite of the misleading history.

Case 4.—Among a herd of 650 hogs, a lot of 40, 6-month-old pigs showed anorexia and incoördination in the hindquarters. All of them had been vaccinated under state supervision when 8 weeks old. Of 2 live specimens submitted, 1 showed petechiation of the spleen, the other of the kidney, and both showed microscopic encephalitis. Revaccination of the remaining herd with serum and virus brought the outbreak under control. In the following year, a single animal representing the first loss was submitted under similar circumstances and revaccination was advised.

Case 5.—In a recent outbreak, a herd of 200 pigs, owned by a correctional institute, which does not practice garbage feeding, suffered a mortality of 7 adult hogs during the preceding month. All animals were regularly vaccinated for hog cholera and hemorrhagic septicemia when 8 weeks old. The affected animals had been treated with hog-cholera antiserum alone, penicillin, and sulfonamides without avail. The 3 live specimens received (1 adult sow and 2, 10-week-old pigs) showed gross lesions suggestive of hog cholera. The diagnosis was substantiated in all 3 instances by the demonstration of encephalitic lesions. Bacteriologic examination was noncontributory. Revaccination with serum and virus of all animals immunized more than eight months ago was advised.

SUMMARY

The value of neuropathologic examination in the differential diagnosis of porcine diseases is discussed. Gross, microscopic, and bacteriologic, and, occasionally, animal inoculation tests on 124 diseased and normal specimens submitted during the past eleven years were supplemented by histologic examination of the brain at five cross-sectional levels.

Of 35 normal swine, 6- to 360-days old, 24 showed under, or near, the ependyma of the lateral ventricle both loose and compact cell aggregates which were found to be primarily composed of oligodendroglia and thus of ectodermal origin. These foci seemed to decrease in number and intensity with advancing age and to have no pathologic significance.

Of 31 uncomplicated hog-cholera specimens, the gray and white matter of the brain showed, in 28 instances, mesodermal reactions consisting of vascular and perivascular infiltrates, microgliosis, capillary hemorrhages, and leptomeningeal infiltrates, indicative of nonsuppurative encephalitis.

Of 13 hog-cholera cases complicated by salmonellosis, pasteurellosis, etc., on the basis of bacteriologic findings, 12 showed similar encephalitic lesions.

Of 23 cases presenting noninfectious disorders and 22 affected with various infectious diseases, only 4 cases showed isolated, barely recognizable, vascular infiltrates of no diagnostic importance.

Recognized infectious entities, such as erysipelas, pasteurellosis, salmonellosis, and hemorrhagic dysentery, which must be considered in the differential diagnosis of hog cholera, and successful hog-cholera vaccination, failed to induce the characteristic neuropathologic lesions.

Encephalitic changes have been described in Aujeszky's disease, Teschen disease, rabies, and listeriosis, none of which have been recognized in Connecticut. These diseases are believed to have been ruled out in this study on the basis of anamnesis, bacteriology, and animal inoculation tests. Thus, the encephalitic changes were interpreted as indicating hog cholera, which diagnosis was supported by virus isolation in 1 subtypical case, and by field observations on the effectiveness of simultaneous hog-cholera revaccination.

Neuropathologic examination is considered to be a valuable aid in the differential diagnosis of porcine diseases.

References

[1]Biester, H. E., and Schwarte, L. H.: Listerella Infection in Swine. J. A. V. M. A., *96*, (1940):329-342.

[2]Bodian, D.: Poliomyelitis. Neuropathologic Observations in Relation to Motor Symptoms. J. Am. M. A., *134*, (1947):1148-1154.

[3]Boynton, W. H., Wood, Gladys H., Wood, F. W., and Casselbery, N. H.: Cell Changes in the Gall Bladder as an Aid in the Diagnosis of Hog Cholera.

Proc. 45th Ann. Meeting U. S. Livestock Sanitary A., (1941):44-47.

[1]Brunschwiler, K.: Ueber Meningitis acuta und verwandte Zustände beim Schwein. Ztschr. f. Infektionskr., *28*, (1925) :277-294.

[2]Diernhofer, K.: Die ansteckende Schweinelähmung. Deutsche tierärztl. Wchnschr., *48*, (1940): 213-215.

[3]Eberdeck, E.: Zur Pathologie der Virusschweinepest. Arch. f. wissensch. prakt. Tierheilk, *63*, (1931): 371-400.

[4]Fekete, L.: Ueber bemerkenswerte unter lyssaähnlichen Symptomen verlaufende Fälle von akuter Schweinepest. Ztschr. f. Infektionskr., *32*, (1927) :63-68.

[5]Fortner: Experimentelle Untersuchungen über die ansteckende Schweinelähme. Deutsche tierärztl. Wchnschr., *3*, (1941):43-44.

[6]Freeman, W.: Neuropathology. W. B. Saunders and Co., Philadelphia, Pa., (1933):43-45.

[7]Gifford, Rebecca, and Jungherr, E. L.: Listeriosis in Connecticut with Particular Reference to a Septicemic Case in a Wild Raccoon. Cornell Vet., *37*, (1947):39-48.

[8]Graf, A.: Histologische Untersuchungen zur Frage des encephalitischen Symptomenkomplexes beim Schwein mit besonderer Berücksichtigung der chronischen Schweinepest. Deutsche tierärztl. Wchnschr., *17*, (1937) :281-282.

[9]Grau, H.: Zur Diagnosestellung bei ansteckender Schweinelähme in den Veterinäruntersuchungsämtern. Berl. Münch. Wchnschr., *8*, (1941):85-88.

[10]Hirt, G.: Histologische Veränderungen des Zentralnervensystems bei der Aujeszkyschen Krankheit. Arch. f. wissensch. prakt. Tierheilk., *70*, (1936): 323-339.

[11]Huguenin, F.: Schweiz. Arch. f. Tierheilk., *65*, (1923). Original not seen. See Seifried.[39]

[12]Hurst, E. W.: Studies on Pseudorabies. J. Exptl. Med., *58*, (1933) :415-433.

[13]Hutyra, F., Marek, J., and Manninger, R.: Special Pathology and Therapeutics of the Diseases of Domestic Animals. Alexander Eger, Inc., Chicago, Ill., 5th English ed., (1946) :286-312.

[14]Joest, E.: Spezielle pathologische Anatomie der Haustiere. Richard Schoetz, 2nd ed. 2 B., Berlin, Germany, (1937) :639-644.

[15]Johannsen, A.: Sektionsbilden vid svinpest i Sverige under epizootierna aren 1940 och 1943. Skand. Vet.-tidskr., *34*, (1944) :467-494.

[16]Kaplan, M. M., and Meranze, D. R.: Porcine Virus Encephalomyelitis and Its Possible Biological Relationship to Human Poliomyelitis. Vet. Med., *43*, (1948) :330-340.

[17]Kernkamp, H. C. H.: Lesions of Hog Cholera: Their Frequency of Occurrence. J. A. V. M. A., *95*, (1939) :159-166.

[18]Kernkamp, H. C. H., and Femtermacher, R.: Subtypical Hog Cholera. Proc. 51st Ann. Meeting, U. S. Livestock Sanitary A., (1947) :96-99.

[19]King, W. E., and Wilson, R. H.: Studies of Hog Cholera and Preventive Treatment. Kansas State Agric. Coll. Exper. Sta., Bull. 171, 1910.

[20]Köbe, L., and Schmidt, W.: Differential-Diagnose zwischen chronischer Schweinepest und Ferkelgrippe. Deutsche tierärztl. Wchnschr., *16*, (1934) :145-148.

[21]Laumen, H.: Untersuchungen über die Lokalisation des Entzündungsprozesses im Gehirn bei Schweinepest. Inaug-Diss. Greifswald, 1931.

[22]Lewis, P. A., and Shope, R. E.: The Blood in Hog Cholera. J. Exptl. Med., *50*, (1929) :719-736.

[23]Lüttschwager: Zur histologischen Diagnose der Virusschweinepest. Arch. f. wissensch. prakt. Tierheilk., *64*, (1932) :236-244.

[24]Manninger, R.: Untersuchungen über Schweinepest und Ferkel Paratyphus. Deutsche tierärztl. Wchnschr., *40*, (1932) :113-120.

[25]McClung, E. E.: Handbook of Microscopical Technique. Oxford Univ. Press. 2nd ed., (1932) :511.

[26]Pinkerton, H. E.: *Salmonella Choleraesuis* or Suipestifer Septicemia. Fort Dodge Bio-Chemic Rev., *25*, (1944) :3-5, 20-22.

[27]Hanson, S. W.: The Anatomy of the Nervous System. W. B. Saunders Co., Philadelphia, Pa., (1936) :56-57.

[28]Ray, J. D.: Pseudorabies (Aujeszky's Disease) in Suckling Pigs in the United States. Vet. Med., *38*, (1943) :178-179.

[29]Rivers, T.: Viral and Rickettsial Infections of Man. J. B. Lippincott Co., Philadelphia, Pa. (1948): 163-208.

[30]Röhrer, H.: Histologische Untersuchungen bei Schweinepest. Veränderungen im Zentralnervensystem in akuten Fällen. Arch. f. wissensch. prakt. Tierheilk., *62*, (1930) :439-462.

[31]Röhrer, H.: Zur histologischen Diagnose der akuten Schweinepest. Deutsche. tierärztl. Wchnschr., *47*, (1939) :733-734.

[32]Röhrer, H., and Graf, A.: Die Bedeutung des selbständigen encephalitischen Symptomenkomplexes für die histologische Diagnose der chronischen Schweinepest. Deutsche. tierärztl. Wchnschr., *40*, (1934) :637-643.

[33]Salvalaglio, P.: Histological Diagnosis of Swine Fever. Clin. Vet., *60*, (1937) :322-324. (Original not seen. Translated title. Abstr. Vet. Rec., *50*, (1938): 764-765.)

[34]Salyi, J.: Beitrag zur Pathohistologie der Aujeszkyschen Krankheit. Arch. f. wissensch. prakt. Tierheilk., *69*, (1935) :55-60.

[35]Seghetti, L.: Observations Regarding *Salmonella Choleraesuis* (var. kunzendorf) Septicemia in Swine. J. A. V. M. A., *109*, (1946) :134-137.

[36]Seifried, O.: Histological Studies on Hog Cholera. J. Exptl. Med., *53*, (1931) :222-289.

[37]Seifried, O.: Die Ausbreitung der "encephalitischen Reaktion" bei Schweinepest und deren Beziehungen zur Hundestaupe-Encephalitis. Arch. f. wissensch. prakt. Tierheilk., *64*, (1932) :432-443.

[38]Seifried, O.: Lehrgang der Histopathologie. Julius Springer, Berlin, Germany, (1934) :139-140.

[39]Shahan, M. S., Knudson, R. L., Seibold, H. R., and Dale, C. N.: Aujeszky's Diseases (Pseudorabies). North Am. Vet., *28*, (1947) :511-521.

[40]Udall, D. H.: The Practice of Veterinary Medicine. Published by author, Ithaca, N. Y., (1943): 464-473.

Clinical and Pathologic Effects Produced in Goats by the Ingestion of Toxic Amounts of Chlordan and Toxaphene

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DURING World War II, the importance of arthropod ectoparasites was greatly emphasized. The fact that many of these were vectors of disease-producing agents caused the most concern. This factor demanded intensive research efforts from many sources. Research workers synthesized new compounds, reëxamined existing formulations, evaluated their efficiency as insect toxicants, and tested their relative safety to the host. Two of these compounds which showed particular promise as insect toxicants were chlordan and toxaphene (chlorinated hydrocarbons).

The object of this experiment is to ascertain the lesions produced by chlordan and toxaphene by administering lethal amounts of each of them orally. Such investigation seems particularly desirable at this time. Little information is available on the pathologic lesions produced in ruminants by these chemicals; and their use is increasing annually. These are available for use either as emulsions or as wettable powders.[1, 14, 20]

The damage done to livestock by older insect toxicants in the control of external parasites was considerable.[10] The discovery and the discriminate use of these newer insect toxicants promise to lower these losses considerably; but in evaluating the usefulness of these new agents to the livestock industry, their shortcomings as well as their advantages must be considered.

REVIEW OF LITERATURE

Chlordan.—Chlordan[9] is toxic to a wide range of Acarina attacking human beings and domestic animals with relatively little harm to warm-blooded animals. Its application on soil protected poultry and other birds from lice for a period of four to five weeks.[5]

Ingle[7] experimented on the toxicity of chlordan to white rats. It was slightly more harmful to female than male rats. When administered intragastrically in lethal doses, the rats died with the symptoms of inappetence, loss of weight, hyperexcitability, rapid respiration, pronounced tremors, and convulsions at twenty- to thirty-minute intervals. A marked increase in respiration within five minutes of application (stomach or skin) was observed.

Grossly, there were hyperkeratosis, brown discoloration, and ulceration of skin following external application. Postmortem lesions were pallor and centrolobular necrosis in the liver, pitting and hemorrhage in the kidneys, and edema and hemorrhage in the lungs.

A dose of 700 mg. of wettable powder of chlordan per kilogram given to dogs produced clonic spasms, tremors, and convulsions without a fatal termination.[21] No symptom was observed in dogs dipped in 0.42 per cent chlordan solution. It was an efficient insecticide, with no apparent detrimental action on the host, when applied in proper concentration.

Radeleff[18] sprayed sheep and goats with 1.5 per cent wettable powder of chlordan every fourth day. He also sprayed cattle with 2.0 per cent wettable powder of chlordan at an interval of a fortnight. These procedures produced clinical nervous manifestations and death.

There was a sudden onset of symptoms. The animals were down and unable to rise. There were groaning, violent paddling of limbs, grinding of teeth, opisthotonos, cyanosis, and death.

Autopsy findings were petechiae in the subepicardium and subserous ecchymosis in the small and large intestines. There were enlargement of the liver, erosion of gastric mucosa, splenic enlargement, excess of peritoneal effusion, and gastroenteritis. Occasionally, the lungs failed to collapse when the thorax was opened.

The histopathologic examination was incomplete but revealed fatty changes in the liver, with central and midzonal lobular necrosis in 1 sheep. In 1 goat, fat deposits were observed in the convoluted tubules of the kidneys. In the brain of some goats, neuron involvement, congestion, and hemorrhage were noted, whereas, in others, the brain showed congestion only. It was observed that the symptoms were similar to those of sodium chloride, chloroform, santonin, cocklebur, and DDT poisoning.

Welch[22] reported that a daily dose of more than 0.77 Gm. of chlordan was apparently injurious after several days' administration.

From the Department of Veterinary Pathology and Bacteriology, A. and M. College of Texas, College Station. An abstract of a thesis presented in partial fulfillment of the requirements for the degree of master of science, A. and M. College of Texas, 1948.

Graduate student in the Department of Veterinary Pathology and Bacteriology (Choudhury); professor of veterinary pathology and bacteriology (Robinson).

Toxaphene.—Investigations on the effect of toxaphene on mammals are reported to be in progress. Toxaphene seemed to be more detrimental when applied to the skin of white rats dissolved in deodorized kerosene than in corn oil.[14]

Dogs[5] suffered from convulsions at the dosage of 20 mg. of toxaphene per kilogram of live weight.

Bishop and Knipling[7] express an urgent need for research, as the availability of data on these newer insect toxicants is limited.

MATERIALS AND METHODS

The object of this experiment was to determine the symptoms and gross and histopathologic lesions which a known lethal quantity of chlordan or toxaphene might produce in goats. No attempt was made to grade the toxicity of these compounds in any manner. The experimental procedure was as follows:

1) Three goats were administered chlordan orally in gelatine capsules.

2) Three goats were given toxaphene orally in gelatine capsules.

3) Doses were calculated in milligrams per kilogram of live weight by weighing each goat at the beginning and every alternate day of treatment.

4) Attempts were made to begin with doses large enough to be toxic but no so large as to kill rapidly.

The doses were gradually increased every second day until symptoms appeared and death occurred in the treated animals. The doses of both the compounds were calculated as shown in tables 1 and 2.

The dearth of data on toxaphene necessitated the administration of as small an initial dose as 25 mg. per kilogram of the body weight to the first goat. It was hoped that the development of lesions would be gradual enough to favor maximum pathology.

5) Forty per cent of the active ingredient in the nontoxic diluent (attaclay) of both chlordan and toxaphene was used in the experiment.

6) One healthy goat was sacrificed to obtain specimens of normal tissues for comparative study.

OBSERVATIONS AND RESULTS OF EXPERIMENT

The symptoms were observed closely and recorded individually. Similarly, they were autopsied as early as possible for accurate macroscopic and histopathologic examinations of lesions in various vital organs of the body.

CHLORDAN

Twenty-four hours after the first dose, the following symptoms were observed: dullness, partial loss of appetite, hyperesthesia to touch or loud noises, slightly dilated pupils, and oc-

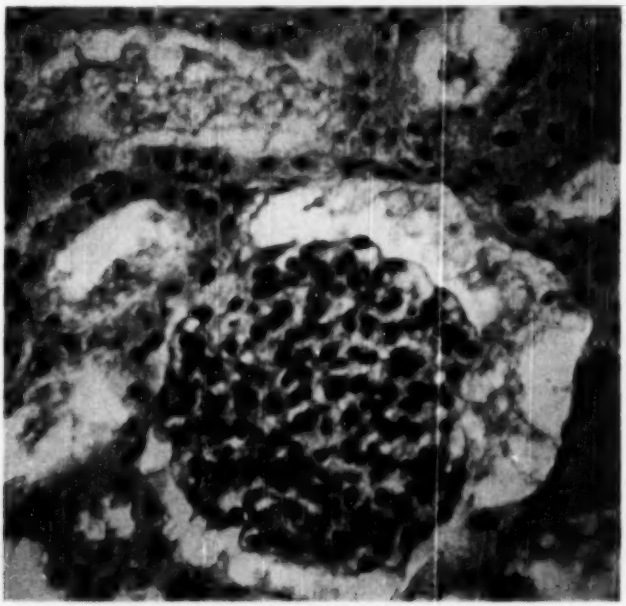

Fig. 1—Section of kidney showing acute glomerulonephritis in goat 3 poisoned with chlordan. x 830.

casional groaning or bleating. After forty-eight hours, there were complete anorexia, slight loss of weight, hunched appearance, and tucked-up flanks. Widely dilated pupils were evident in goats 1 and 3. The animals manifested restlessness, staggering gait, muscular stiffness of the hind legs, circling (in goat 3), grinding of the teeth, frequent attempts at micturition, and increased nervous reflexes. Paroxysms of convulsions, lasting two to three minutes or more, usually resulted when attempts were made to secure them for dosing or examination. During convulsions, they fell to the ground on the side drawing the head towards the back and shaking the same. The legs were either rigid or there was paddling. Sometimes, there were champing of the jaws and discharge of frothy saliva. Respiration was shallow and rapid. Dyspnea was pronounced before death. The convulsions were more frequent as death supervened. The animals became weak, eventually lay down, and were disinclined to move. When urged to move, they usually went into convulsions. Ultimately, they became cyanotic and died. (*See* table 1.)

Macroscopic Appearance.—There was extensive pulmonary edema evidenced by a large quantity of frothy exudate which seemingly filled the alveoli, bronchioles, and bronchi and partially occluded the trachea. There were focal areas of pulmonary emphysema and the lungs failed to collapse on opening the thorax. The pulmonary vessels were engorged with blood and the bronchial lymph nodes were edematous. The right side of the heart was distended and there was an excess of pericardial fluid.

The abomasum exhibited irregular areas of congestion. These were large, small, or splotchy and were more pronounced near the pyloric orifice.

The small intestine was congested throughout. The presence of catarrhal and hemorrhagic inflammation of the mucosae was evident. The mucosa was coated with a tenacious exudate tinged with blood and eroded in places. The mesenteric lymph nodes were swollen.

The liver was hyperemic, swollen, friable, and glossy. Ecchymosis of the gall bladder of goats 1 and 3 was noticed.

The kidneys showed irregular hemorrhagic areas on the cut surface continuous with the cortex and medulla.

The blood vessels of the meninges and brain were distended and there was an excess of cerebrospinal fluid. The brain and spinal cord were slightly softened where sliced. There was nothing indicative of cerebrospinal hemorrhage.

Histopathology.—Brain and Spinal Cord.—The blood vessels in the brain and spinal cord were engorged with blood. Perivascular edema, capillary thrombosis, and migration of a number of round cells were noted throughout the brain. The nerve cells and fibers showed degenerative changes, in some cases to the point of disintegration. There were small

TABLE 1—Results of Feeding Goats with Chlordan (40 Per Cent Wettable Powder)

Serial No. of goat	Day of experiment	Age of animal	Live weight (kg.)	Active ingredient in mg. per kg. of body weight	Total quantity of active ingredient (mg.)	Total quantity of 40% preparation of the active ingredient given	Clinical results
1	1	1 yr.	17.95	750.00	13,462.50	33,656.25	No effect.
	2	1 yr.	17.95	750.00	13,462.50	33,656.25	Hunched up appearance, dullness, dilated pupil, occasional groaning or bleating, slightly increased reflex, inappetence, and rapid respiration.
	3	1 yr.	17.50	750.00	13,125.00	32,812.50	Complete anorexia, loss of weight, staggering gait, muscular stiffness of hind quarters, restlessness, increased reflex, convulsion, dyspnea, asphyxia, death at the 70th hour.
3	1	2 yr.	22.00	750.00	16,500.00	41,250.00	No effect.
	2	2 yr.	22.00	750.00	16,500.00	41,250.00	Symptoms were similar to those of goat 1.
	3	2 yr.	20.45	750.00	15,337.50	38,343.75	Symptoms were similar to those of goat 1. Circling was noticed after 48 hours, died at the 68th hour.
4	1	1 yr.	20.00	750.00	15,000.00	37,500.00	Slight nervous irritability.
	2	1 yr.	20.00	750.00	15,000.00	37,500.00	Symptoms were similar to those of goats 1 and 3 but more severe. Died at the 28th hour.

areas of softening and edema in the gray and white matter. The degeneration of neurons and ganglion cells was accompanied by neuronophagia by microglia type cells.

Heart.—There was edema of the muscular septums and cloudy swelling of muscle cells with a tendency to fatty metamorphosis. Moderate focal and diffuse neutrophilic and round cell infiltration was observed throughout the myocardium. There were edema and neutrophilic infiltration in the subendocardial space.

Lungs.—The lungs were edematous, congested, and hemorrhagic. Most of the alveoli, bronchioles, and bronchi were filled with exudate, rich in protein, desquamated epithelium, round cells, and neutrophils. Some were either emphysematous or atelectatic. There was a marked neutrophilic and round cell infiltration. The interalveolar septums were thickened and edematous except in some areas of emphysema. A considerable amount of hemosiderin was present.

Lymph Nodes.—The mesenteric and bronchial lymph nodes were congested and contained hemosiderin. Changes were more severe in the bronchial nodes.

Spleen.—The changes were more or less similar to those found in the lymph nodes. The reticulo-endothelial cells were proliferated. Phagocytosis of cell débris, mild neutrophilia, and hemosiderosis were present, with numerous chromatin fragments.

Adrenals.—Infiltration of a few round cells, congestion, slight hemorrhage, edema, and cloudy swelling were noticed both in the cortex and medulla.

Kidneys.—The acute glomerulonephritis and changes in the tubular epithelium were most outstanding. The glomeruli were distended with sloughed epithelial cells and coagulated protein. Occlusion of many uriniferous tubules, with cellular and granular casts, was evident. This resulted in the distention of many of the proximal tubules and Bowman's space. Both cortical and medullary portions were congested and round cells had invaded the interstitial tissue. The tubular epithelium had undergone cloudy swelling, fatty metamorphosis, coagulation necrosis, and desquamation. The intertubular capillaries were distended with blood which compressed the tubules on either side. At different points, the vessels gave way and the red blood corpuscles were lodged in the interstices or in the tubular lumen. The endothelial cells of the capillary tufts in the glomeruli were multiplied, swollen, and completely filled Bowman's space, in many instances. Some vacuoles were formed in the capillary tufts and protein accumulated in them.

Abomasum.—Congestion, edema, round cell infiltration, and mild excoriation of mucous membrane were observed.

Small Intestine.—All layers of the small intestine were swollen, edematous, and infiltrated with many round cells, some neutrophils, and plasma cells. Some capillaries of the muscularis mucosae were plugged with thrombi. The mucous membrane was swollen, excoriated, and hemorrhagic. The edema was mostly restricted to the submucous layer. The mucous surface was covered with a catarrhal exudate, containing desquamated epithelium, round cells, neutrophils, plasma cells, and mucin. The capillaries were engorged and the tips of the villi sloughed off.

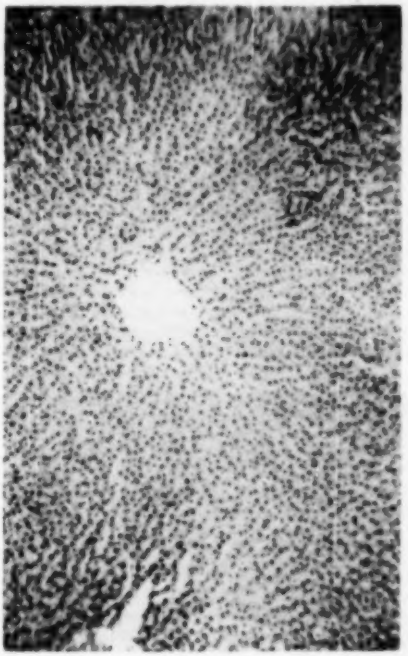

Fig. 2—Section of liver showing acute hepatitis in goat 3 poisoned with chlordan. x 160.

Liver.—Acute hepatitis with infiltration of round cells, cloudy swelling, fatty metamorphosis, and focal coagulation necrosis was present. Vacant spaces, around the central veins, caused by necrosis of the hepatic cells, were filled in with protein fragments of the degenerated cells. Some of the hepatic cells shrank, which resulted in the atrophy of the hepatic cord. The atrophy and degeneration of the hepatic cord caused the sinusoid to distend and engorge with blood and cell débris. There was engorgement of the large blood vessels. The endothelial lining of all blood

vessels was swollen and increased in prominence.

TOXAPHENE

At first, there was impaired appetite, later followed by total inanition. There were dilatation of pupils, rapid and superficial respiration, groaning, bleating, grinding of teeth, restlessness, and stiffness in the hind quarters. The flanks were tucked up and they moved with posterior limbs apart. There were excessive wriggling of the tail, frequent attempts to micturate, highly colored urine, and oliguria. Later, nervous irritability increased, startling was produced with the slightest provocation, and convulsions developed. During convulsive fits, the goat was on the ground, the head was turned backwards, and there was paddling of the limbs. Muscular tremors were consistently present. The convulsions lasted two to three minutes. These were followed by relaxation accompanied with marked depression. There was a marked loss of weight due to anorexia, dehydration, and exhaustion. Prior to death, there was labored respiration succeeded by dyspnea, cyanosis, and asphyxia. Death supervened with or without convulsion. (*See* table 2.)

Macroscopic Appearance.—The lungs were edematous, hyperemic, heavy, and firm. The alveoli, bronchioles, bronchi, and trachea contained frothy serous fluid slightly tinged with blood. The mucous membrane was hyperemic.

The heart was enlarged and the epicardium was petechiated along the course of the coronary vessels and about the trigona fibrosa. Pericardial fluid was present.

The mucosa of the abomasum was congested, thickened, and slightly eroded near the pylorus.

The small intestine was congested and hemorrhagic. The mucosa was spotted with erosions and covered with thick exudate, the contents faintly sanguineous. The lesions were more vivid from the posterior end of the jejunum to the ileum, less severe in the large intestine. The mesenteric lymph nodes were hyperemic and enlarged.

The liver was enlarged, hyperemic, and glossy. The gall bladder contained ecchymotic hemorrhages. Irregular large and small areas of congestion on the surface of the kidneys were continuous with the cortex and medulla on the cut surface.

The vessels of the meninges, brain, and spinal cord were markedly injected and distended with blood. There was some evidence of hemorrhage in the brain.

In goat 5, the petechiae in the subepicardium were few in number; whereas, in goat 6, they were numerous.

Histopathology.—Brain and Spinal Cord.—Congestion, edema, infiltration of round cells, and a few neutrophils were found throughout but were especially severe in the medulla.

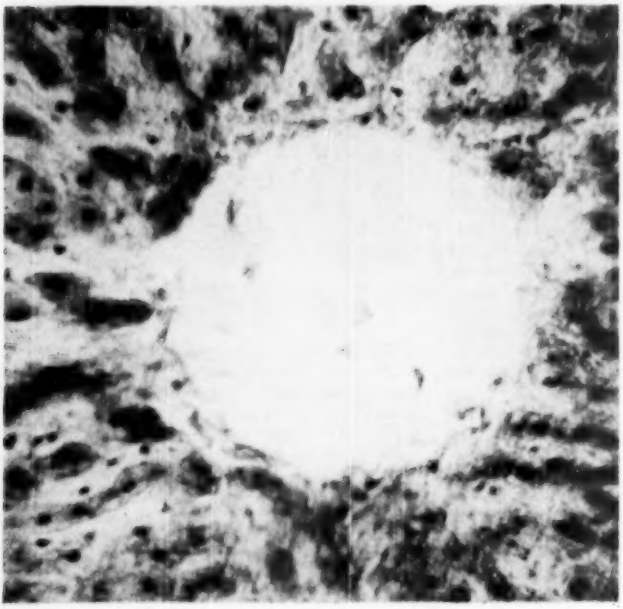

Fig. 3—Section of liver showing distended sinusoid and cellular changes in goat 5 poisoned with toxaphene. x 372.

There were spotted softening and degeneration of neurons in the brain and spinal cord. Obvious neuronophagia existed in all 3 cases. In goat 6, numerous petechial hemorrhages were present in the medulla.

Heart.—Congestion, edema, hemorrhage, round cell and neutrophilic infiltration, cloudy swelling, and fatty metamorphosis of the cardiac muscles were consistent findings. Circumscribed hemorrhages in the subepicardium along the course of the coronary vessels were noted. There were rupture of the muscle cells, apparent escape of myohematin, and consequent pigmentation.

Lungs.—Congestion, edema, hemorrhage, hemosiderosis, and migration of round cells and neutrophils were observed. There were swelling of the alveolar wall, epithelial desquamation, emphysema, and atelectasis Rupture of the alveolar wall, interstitial hemorrhage, and infiltration of round cells and neutrophils were observed. There was interlobular and alveolar edema. The alveoli were plugged with a homogenous exudate which stained pink. Some bronchioles were ruptured and others collapsed. They contained serous exudate and desquamated epithelium. The pulmonary vessels were highly injected and many capillaries were plugged with thrombi.

Lymph Nodes.—The bronchial and mesenteric lymph nodes were congested and swollen. Mononuclear phagocytes were found containing hemosiderin.

Spleen.—There were congestion, possible hemorrhage, swelling, and necrosis of many reticular cells (karyorrhexis). There were perivascular edema and hemosiderosis. The sinuses were engorged with lymphocytes, red blood cells, a few neutrophils, and protein fragments.

Adrenals.—There were congestion, cloudy swelling, scattered focal necrosis, and slight round cell and neutrophilic infiltration. The intercellular spaces were distended with débris and coagulated protein material. Both the medulla and cortex were affected.

Kidneys.—There were marked cortical and medullary congestion, hemorrhage, hemosiderosis, and thrombosis of smaller vessels. The intertubular edema, capillary congestion, and round cell infiltration had resulted in the compression of tubules and consequent distention of the proximal end of some tubules and Bowman's space. The tubular epithelium was undergoing cloudy swelling, fatty metamorphosis, coagulation necrosis, and desquamation.

Most glomeruli were enlarged and packed with desquamated endothelium. There were epithelial desquamation in Bowman's capsule and formation of granular and cellular casts. Numerous vacuoles were formed in the glomerular tufts. There were engorgement and distentions of the tufts but in some there was partial hyalization. Some of the distended Bowman's capsules contained atrophied capillary tufts due to the pressure of regurgitated urine from the occluded tubules. In some instances, the capillary tufts were enlarged to the point of completely filling Bowman's space by proliferation of endothelium, infiltration of neutrophils, and edema. There were localized areas of coagulation necrosis under the renal capsule in the cortex due to anemic infarction.

Abomasum.—The mucous membrane was covered with exudate containing desquamated epithelium, round cells, plasma cells, neutrophils, and mucin. There was an increased activity of the mucus-producing cells. Focal necrosis and complete sloughing of the mucour lining was the most striking feature. There was extensive edema under the muscularis mucosae. Moderate exudation of fluid and infiltration of round cells had occurred in the muscular layer.

Small Intestine.—The intestinal mucosa was covered with catarrhal exudate containing round cells, plasma cells, neutrophils, desquamated epithelium, cell fragments, and mucin. The tips of the villi were sloughed in places, and crypts were filled with inflammatory exudate and mucin. Severe sloughing of the villi was present over wide areas. The mucous membrane was hemorrhagic throughout. There was edema under the muscularis mucosae and in the muscular layers. The edema contained numerous reactive cells.

The micropathology of the cecum was similar to that of the small intestine but, beyond that, it diminished abruptly.

Liver.—Congestion, hemosiderosis, cloudy swelling, fatty metamorphosis, and focal necrosis around the central veins were pronounced. The reticulo-endothelial cells were swollen and increased in number. There was a moderate infiltration of round cells and neutrophils. The sinusoids were distended with shreds of coagulated protein, chromatin, and cell débris. Some of the hepatic cells were shrunken, causing narrowing of the cord and concomitant enlargement of the adjacent sinusoids.

DISCUSSION

This experiment demonstrated that both chlordan and toxaphene were capable of producing death in goats. The harmful effects of these chlorinated hydrocarbons were manifested clinically, macroscopically, and microscopically.

Both chlordan and toxaphene seemed to behave alike in this experiment. The essential difference in the two was the degree of severity of the lesions produced by an individual compound.[2] Goats 1 and 3 withstood three consecutive doses of chlordan (*see* table 1), while goat 4 died a few hours

after the administration of the second dose of the compound, although the doses were the same. A possible reason advanced for this variation was the difference in the acuteness of lesions produced in individual goats.

The lesions produced by chlordan were gastroenteritis (without diarrhea) and severe pulmonary congestion and edema. There were cardiac enlargement, grossly, and endomyocarditis, microscopically. There were congestion and degeneration of the meninges, brain, and spinal cord. Asphyxia, originating from extreme pulmonary congestion and edema, was believed to be the immediate cause of death. The goats appeared to be more readily affected by chlordan than the sheep,[22] as the latter could tolerate a daily dose of 770 mg. per kilogram for seven days, whereas a daily dose of 750 mg. per kilogram was appreciably injurious for goats within forty-eight hours and ended in death within seventy-two hours.

The nature of the resulting lesions was similar in both instances, but it required a lesser quantity (*see* tables 1 and 2) of toxaphene than that of chlordan to produce more severe lesions. These were manifested by a tendency to hemorrhage from the gastrointestinal tract, kidneys, lungs, heart, and brain.

The diseased condition of the intestinal epithelium resulted in abnormal permeability, edema, and hemorrhage. The edema might be attributed partially to disturbed renal function. The pulmonary congestion and the labored efforts for oxygenation of blood accounted for the dilatation of the right side of the heart.

The lesions in the lungs were so marked that they practically overshadowed the

TABLE 2—Results of Feeding Goats with Toxaphene (40 Per Cent Wettable Powder)

Serial No. of goat	Day of experiment	Age of animal	Live weight (Kg.)	Active ingredient in mg. per kg. of body weight	Total quantity of active ingredient (mg.)	Total quantity of 40% preparation of the active ingredient given	Clinical results
2	1	1 yr.	22.27	25.0	556.75	1,391.625	No effect.
	2	1 yr.	22.27	25.0	556.75	1,391.625	No effect.
	3	1 yr.	21.82	25.0	545.50	1,363.750	No effect.
	4	1 yr.	21.82	25.0	545.50	1,363.750	No effect.
	5	1 yr.	21.60	37.5	806.80	2,017.000	No effect.
	6	1 yr.	21.60	37.5	806.80	2,017.000	No effect.
	7	1 yr.	21.50	50.0	1,075.00	2,687.500	No effect.
	8	1 yr.	21.50	75.0	1,612.50	4,031.250	No effect.
	9	1 yr.	20.14	100.0	2,014.00	5,035.000	Stiffness, staggering gait, restlessness, moving with hind legs apart, bleating, groaning, frequent attempts to micturate, high color urine, quick and shallow respiration, dilatation of pupil, increased nervous irritability, convulsions, muscular tremor, paddling of the limbs during convulsion. The symptoms appeared on the 9th day and began to aggravate more and more until it died early in the morning of the 11th day.
	10	1 yr.	20.14	100.0	2,014.00	5,035.000	
	11	1 yr.	18.70	150.0	2,805.00	7,012.500	
5	1	2 yr.	25.00	100.0	2,500.00	6,250.000	No effect.
	2	2 yr.	25.00	100.0	2,500.00	6,250.000	Symptoms were similar to those of goat 2. The goat died toward the end of the 4th day of the experiment. It was seriously sick so the dose of toxaphene was not repeated after the 3rd day.
	3	2 yr.	24.50	150.0	3,675.00	9,187.500	
	4	2 yr.	24.50	.....	.....	.....	
6	1	2 yr.	28.00	150.0	4,200.00	10,500.000	Symptoms were the same as above but ushered in with such a gravity that the goat was extremely sick toward the end of the 1st day and died at the beginning of the second day. The second dose of the compound was not given as that was thought unnecessary.

gross changes of all other organs. Death in every case was seemingly due to a rapid cut in respiratory capacity owing to the pronounced edema. The reduced pulmonary oxidation of blood, in turn, restricted tissue respiration. Thus, the dearth of oxygen seriously hampered the normal function of all the organs and death supervened. This probably accounted for the fatal termination irrespective of convulsion.

The diagnosis of chlordan or toxaphene poisoning must depend on the history, clinical symptoms, and macroscopic and histopathologic changes in various organs, as they produced no specific lesion.

SUMMARY

Chlordan and toxaphene may produce grave symptoms or even death in warm-blooded animals, like all other chemicals used as insect toxicants. The pathogenicity of chlordan and toxaphene includes the gastrointestinal tract, liver, kidneys, suprarenals, heart, lungs, lymph nodes of the affected organs, brain, and spinal cord. The immediate cause of death seems to be retarded oxygenation of blood due to pulmonary edema, emphysema, and atelectasis. The pulmonary lesions are probably coupled with gastroenteritis, hepatitis, nephritic change, carditis, adrenal pathology, and changes in the brain and spinal cord. In other words, these chemicals affect most of the vital organs of the body but pulmonary lesions are the most severe.

The severe renal pathology produced and the suppression of urine production were probably responsible for the further accumulation of these compounds. Toxaphene produces lesions more rapidly than chlordan. Petechial hemorrhage in the epicardial surface of the heart were not observed in goats poisoned with chlordan but were a consistent finding in toxaphene poisoning.

Both chlordan and toxaphene seem to affect especially the capillary endothelium and nervous tissue. Congestion of the meninges, brain, and spinal cord is usually present. Focal miliary softening, necrosis of neurons, perivascular edema, infiltration of round cells, and neuronophagia are constant findings in the central nervous system. The various lesions are manifested clinically by increased reflexes, convulsions, muscular tremors, oliguria, anorexia, anoxia, and death.

References

[1]Anand, P. N.: Memorandum Bur. Entomology and Plant Quarantine, U. S. Dept. Agric. Release, Feb. 28, 1947.

[2]Batte, E. G., and Turk, R. D.: Toxicity of Some Synthetic Insecticides to Dogs. J. Econ. Ento., 41, (1948):102.

[3]Bishop, F. C., and Knipling, E. F.: Insecticides Applied on Livestock. Indust. and Engin. Chem., 40, (1948):713-716.

[4]Boyd, W.: A Textbook of Pathology. Lea and Febiger, Philadelphia, Pa., 5th ed., 1947.

[5]Creighton, J. T., Hetrick, L. A., Hunt, J. P., and Duncan, D. U.: Application of Chlorinated Hydrocarbons to the Soil and Roosts Effectively Controls Lice of Poultry. Poult. Sci., 26, (1947):674-675.

[6]Hutyra, F., Marek, J., and Manninger, R.: Special Pathology and Therapeutics of the Diseases of Domestic Animals. Vol. I-III. Alexander Eger, Inc., Chicago, Ill., 5th English ed., 1946.

[7]Ingle, L.: Toxicity of Chlordan to White Rats. J. Econ. Ento., 40, (1947):264-268.

[8]Ivy, E. E., Farncia, C. R., Jr., and Ewing, K. P.: Chlorinated Camphene for Control of Cattle Insects. J. Econ. Ento., 40, (1947):513-517.

[9]Julius Hyman and Co.: Octa-Klor, the Chlorinated Hydrocarbon Insect Toxicant. 35 pp. Abstr. in J.A.V.M.A., 110, (1947):334.

[10]Julius Hyman and Co.: Eradication of Livestock Parasites: Ticks, Lice, Mange Mites, Fleas, and Flies. Tech. Supplement 203A, Julius Hyman and Co., Denver, Colo., February, 1948.

[11]Kulash, W. M.: Benzene Hexachloride and Chlordan to Control Cotton Boll Weevil. J. Econ. Ento., 40, (1947):644-650.

[12]Maximow, A. A., and Bloom, D.: A Textbook of Histology. W. B. Saunders Co., Philadelphia, Pa., 4th ed., 1947.

[13]Ogilvie, R. F.: Pathological Histology. The Williams and Wilkins Co., Baltimore, Md., 3rd ed., 1947.

[14]Parker, D. L., and Beacher, J. H.: Toxaphene, A Chlorinated Hydrocarbon with Insecticidal Properties. Univ. Delaware, Bull. 264, February, 1947.

[15]Plumb, K. P.: Don't Just Sit There! Do Something: Use Fast Acting Chlordan to Kill Beetle Grubs in Your Lawn. House Beautiful, 90, (1948):217-218.

[16]Radeleff, R. D.: Chlordan Poisoning Symptomology and Pathology. Vet. Med., 43, (1948):342-347.

[17]Shepard, H.: Biological Methods of Testing Insecticides. Indust. and Engin. Chem., 40, (1948):702-704.

[18]Runnells, R. A.: Animal Pathology. Iowa State College Press, Ames, Iowa, 4th ed., 1946.

[19]Stearns, L. A., Parker, W. LeRoy, MacCreary, Donald, and Connell, W. A.: Results with a Chlorinated Bicyclic Terpene (3956) Against Certain Fruit and Vegetable Insects. J. Econ. Ento., 40, (1947):79-83.

[20]Stearns, L. A.: Progress Report on a New Insecticide: Test Result with New Chlorinated Bicyclic Terpene Against Household Insects, Compared with DDT and O.T.I. Soap and San. Chem., 23, (1947):117-119.

[21]Turk, R. D., and Batte, E. G.: Chlordan in the Control of External Parasites of Dogs and Cats. North Am. Vet., 29, (1948):359-360.

[22]Welch, H.: Tests of the Toxicity to Sheep and Cattle of Certain of the Newer Insecticides. J. Econ. Ento., 41, (1948):36-39.

Observations on the Anthelmintic Value of Substituted Phenolic Compounds in Dogs

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THE WRITER has undertaken the evaluation of a large variety of compounds as anthelmintics because it is evident from the information at hand that most of the present drugs have serious limitations. The results obtained to date with a series of substituted phenols form the subject of this report.

The early remedies used for the removal of the macroparasites from man and domestic animals were chiefly derived from plants. Among these botanical products was the phenolic compound, thymol, first introduced by Bozzolo[1] in 1881, and subsequently used to a great extent for the removal of hookworms in man. Since this work, other contributions published have discussed the use of phenolic compounds as anthelmintics, but comparatively little information of a fundamental nature appeared before the investigations of Lamson and his coworkers,[2,3,4,5,6] who studied the anthelmintic properties of a large variety of compounds. Enzie[7,8] recently studied a series of phenolic compounds, including several which were investigated by Lamson *et al.*, in an attempt to determine the usefulness of such products for the control of worms in domestic animals. He reported that a number of these phenols appeared to be of value for this purpose.

MATERIALS AND METHODS

The chemicals investigated included mono-, di-, tri-, tetra-, and pentasubstituted phenols and were obtained for the most part from the research laboratories of Koppers Company, Inc. These phenols were administered to dogs in an unaltered state in hard gelatin capsules at various dose levels. No purgatives or other supplementary treatments were used in these experiments.

The structural formulas, the physical state, and the melting or boiling points of the compounds used in these investigations are listed in table 1. All of these compounds are insoluble in water or only slightly so.

The dogs employed as test animals were of mixed breeds, varying in age from 2 months to 1 year. The experimental animals were placed in individual cages and all feces were collected for two or three days before administration of the drug, in order to determine the extent to which helminths were being spontaneously eliminated and the approximate level of infection. In every instance, the dogs were fasted for a period of eighteen to twenty-four hours before treatment. The response of the dogs to the drug at the time of administration and for the following three days was observed. During this interval, all stools were again collected. After these observations, the dogs were sacrificed and examined for pathologic changes. The entire contents of the digestive tract were washed through various-sized screens and all worms present were collected, identified, and counted. The feces collected after treatment were also screened and the worms which were isolated were combined with those obtained from the digestive tract to establish the number present at the time of treatment. The percentage of efficacy of the drug was determined on the basis of the total number of worms passed following treatment, as represented by the number of expelled worms plus the living parasites isolated at necropsy.

RESULTS

For convenience in discussion of the biologic results observed, the phenolic materials investigated have been combined into four groups: (1) mono-, (2) di-, (3) tri-, and (4) tetra- and pentasubstituted phenolic compounds. The results obtained with the six monosubstituted phenols are summarized in table 2. Dodecylphenol, N-acetyl-2-aminophenol, and N-benzyl-4-aminophenol were relatively ineffective. However, it will be noted in the table that three of the phenols showed evidence of anthelmintic action. These included 4-tert.-butylphenol, mixed sec. and tert.-2-amylphenol, and indanylphenol. In most cases, the administration of these compounds caused no noticeable reaction in the host, and none of the test animals presented pathologic changes in the internal organs.

The chemical 4-tert.-butylphenol, reported by Enzie[7] to be effective for the removal of ascarids, varied in its efficacy in these

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The author is deeply indebted to Mr. H. G. Guy for his assistance in selecting the compounds used in this study.

TABLE I—Substituted Phenolic Compounds Investigated

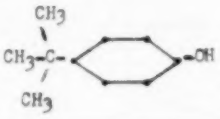
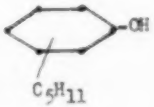
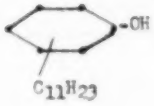
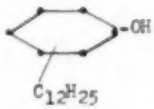
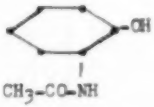
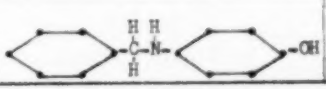
NAME	FORMULA	PHYSICAL STATE ROOM TEMP.	BOILING OR MELTING POINT
	MONOSUBSTITUTED PHENOLS		
4-Tert.-butyl-phenol		Solid	M.P. 96-100°C
Mixed-2-amyl-phenol (Secondary and Tertiary)		Liquid	B.P. 175-185°C (75 mm.)
Indanylphenol		Solid	B.P. 165-200°C (2.4 mm.) (Mostly 180-185
Dodecylphenol		Liquid	B.P. 120-179°C (1.5 mm.)
N-acetyl-2-aminophenol		Solid	M.P. 201-203°C
N-benzyl-4-aminophenol		Solid	M.P. 86-88°C

TABLE I (continued)—Substituted Phenolic Compounds Investigated

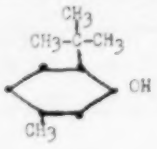
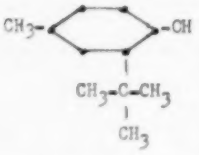
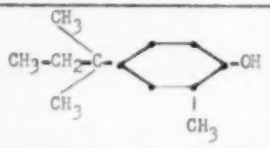
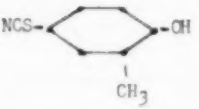
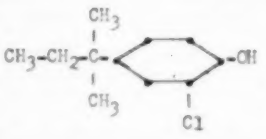
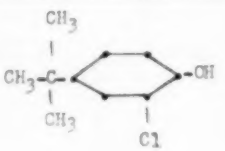
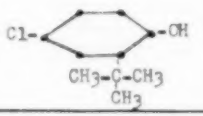
NAME	FORMULA	PHYSICAL STATE ROOM TEMP.	BOILING OR MELTING POINT
	DISUBSTITUTED PHENOLS		
6-Tert.-butyl-m-cresol		Liquid	B.P. 244°C M.P. 20-23°C
2-Tert.-butyl-p-cresol		Solid	B.P. 124°C (20 mm.)
P-Tert.-amyl-o-cresol		Liquid	B.P. 260-261°C
Thiocyano -o-cresol		Solid	Technical
4-Tert.-amyl-2-chlorophenol		Liquid	B.P. 253-265°C
4-Tert.-butyl-2-chlorophenol		Liquid	Technical
2-Tert.-butyl-4-chlorophenol		Liquid	B.P. 140°C (20 mm.)

TABLE I (continued)—Substituted Phenolic Compounds Investigated

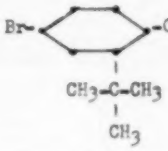
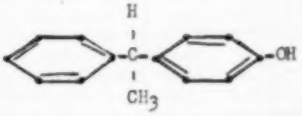
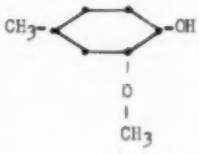
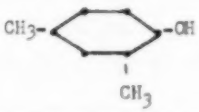
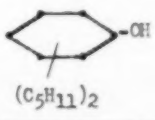
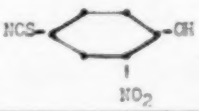
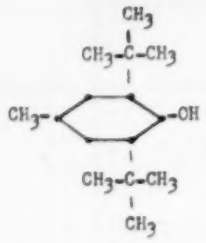
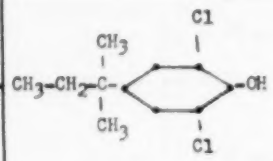
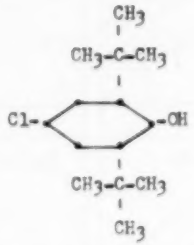
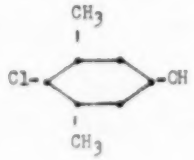
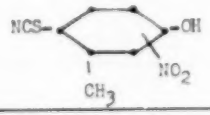
NAME	FORMULA	PHYSICAL STATE ROOM TEMP.	BOILING OR MELTING POINT
	DISUBSTITUTED PHENOLS		
2-Tert.-butyl-4-bromophenol		Liquid	B.P. 137°C (10 mm.)
Phenylethyl-phenol		Semi-solid	B.P. 150°C (1 mm.)
2-Methoxy-4-methylphenol		Solid	Technical
2,4-Dimethyl-phenol		Liquid	M.P. 20-22°C
Dianylphenol		Liquid	B.P. 275-310°C
Nitrothiocyano-phenol		Solid	Technical

TABLE I (continued)—Substituted Phenolic Compounds Investigated

NAME	FORMULA	PHYSICAL STATE ROOM TEMP.	BOILING OR MELTING POINT
	TRISUBSTITUTED PHENOLS		
2,6-Ditert.-butyl-p-cresol		Solid	Technical
4-Tert.-amyl-2,6-dichlorophenol		Solid	B.P. 155-170°C (15 mm.)
2,6-Ditert.-butyl-4-chlorophenol		Solid	B.P. 162°C (20 mm.)
3,5-Dimethyl-4-chlorophenol		Solid	M.P. 114-117°C
4-Thiocyano-3-methyl-nitrophenol		Solid	Technical

experiments when tested at different dose levels on a number of dogs. When given 0.2 Gm. per pound of body weight, this phenol removed 33 of 42 ascarids in 1 dog and all ascarids in 2 other dogs. When given 0.1 Gm. per pound of body weight, 22 to 100 per cent of the ascarids were removed in 4 dogs. In general, these results are poorer than those reported by Lamson[6] and Enzie[7] but tend to confirm their findings.

The mixed monoamylphenol used in these experiments was a technical product and contained, for the most part, 2-sec.-amylphenol plus a small amount of 2-tert.-amylphenol. It is a clear liquid and has a slightly irritating action when placed on the oral mucosa. Upon necropsy, no pathologic changes were noted in any of the test animals when given 0.1 cc. per pound of body weight. At this concentration, this compound removed all of the ascarids from 4 dogs and all of the hookworms from 2 dogs (table 2). Neither this phenol nor any of the others investigated were effective in removing whipworms, possibly because of the location of this parasite in the lower digestive tract. Nevertheless, this amylphenol should be considered as promising and worthy of further study on dogs and other animals.

Indanylphenol expelled 100 per cent of the ascarids from 2 dogs given 0.1 Gm. per pound of body weight but was ineffective against hookworms and whipworms. At 0.05 Gm. per pound of body weight, this phenol was relatively ineffective against ascarids.

The remaining three monosubstituted phenols, as already indicated, seemed to have very little value as anthelmintics. A limited number of tapeworms were present in a few of the host animals. None of these was removed by these monosubstituted phenols. The amylphenol, however, has not as yet been administered to an animal infected with tapeworms.

Seven of the 13 disubstituted phenols included in these studies were effective in removing helminths from dogs (table 3). The compound 6-tert.-butyl-m-cresol was found effective in the removal of ascarids over a range of concentrations. When administered in a dose of 0.3 cc. per pound of body weight, the dogs vomited three hours after receiving the drug. Nevertheless, 100 per cent of the ascarids were removed, but the hookworms present were apparently unaffected. Of the hookworms, 84 per cent were removed by a dose of 0.1 cc. per pound of body weight, but in this instance no vomiting occurred. There were no reactions by the dogs and no evidence of pathologic change was found at necropsy. This compound is under further investigation at the present time, and it is planned to present the results of these studies in a later publication.

The compound 2-tert.-butyl-p-cresol, differing chemically from the previous compound only in the position of the substituent groups on the phenolic ring, was also

TABLE I (continued)—Substituted Phenolic Compounds Investigated

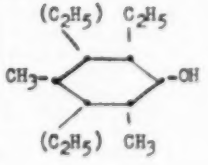
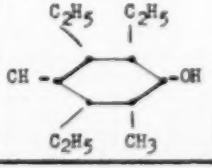
NAME	FORMULA	PHYSICAL STATE ROOM TEMP.	BOILING OR MELTING POINT
TETRA- AND PENTASUBSTITUTED PHENOLS			
2,4-Dimethyl-(3,5)6-diethylphenol		Liquid	B.P. 147°C (20 mm.)
2,4-Dimethyl-3,5,6-triethylphenol		Liquid	B.P. 168°C (20 mm.)

TABLE 2—Anthelmintic Value of Monosubstituted Phenol Compounds

Compound	Exp. (No.)	No. dogs used	Dose per lb. body wt.	Post-treatment fecal exam.	Autopsy findings	Efficiency (%)	Remarks
4-Tert.-butylphenol	32	2	0.2 Gm.	5 ascarids	240 whipworms	100 0	No reaction noted.
	35	2	0.2 Gm.	43 ascarids 1 whipworm	9 ascarids 150 whipworms	82 0	No reaction noted.
	245	2	0.1 Gm.	11 ascarids	9 ascarids	55	No reaction noted.
	247	2	0.1 Gm.	12 ascarids	1 ascarid 15 whipworms 5 Dipylidium	92 0 0	No reaction noted.
Mixed-2-amylphenol	252	2		[illegible] ascarids [illegible] hookworms		100 100	No reaction noted.
					7 whipworms	0	
	276	2	0.1 Gm.	26 ascarids		100	No reaction noted.
					7 whipworms	0	
Indanylphenol	261	2	0.1 Gm.	39 ascarids		100	2 ascarids expelled preceding treatment.
					1 hookworm	0	No reaction noted.
					14 whipworms	0	
	266	2	0.05 Gm.			--	2 ascarids expelled preceding treatment.
				1 hookworm	58 hookworms	0	No reaction noted.
						0	
Dodecylphenol	148	2	0.1 Gm.	2 ascarids	12 ascarids	15	No reaction noted.
					18 hookworms	0	
					24 whipworms	0	
N-Acetyl-2-aminophenol	53	4	0.1 Gm.	1 ascarid	5 ascarids	16	No reaction noted.
					1 whipworm	0	
					79 Dipylidium	0	
	121	4	0.1 Gm.	7 ascarids	15 ascarids	32	2 ascarids expelled preceding treatment.
					2 hookworms	0	No reaction noted.
					7 whipworms	0	
					15 Dipylidium	0	
N-Benzyl-4-aminophenol	52	2	0.1 Gm.	7 ascarids	7 ascarids	50	No reaction noted.
					1 hookworm	0	
					15 whipworms	0	
					3 Dipylidium	0	
					3 Taenia	0	

found to be effective against ascarids when administered in a dose of 0.1 to 0.2 Gm. per pound of body weight. In these tests, this material also eliminated the tapeworms present but appeared to be ineffective against hookworms. It would appear that 2-tert.-butyl-p-cresol is toxic to dogs when given at the rate of 0.3 Gm. per pound of body weight; both animals died within seventeen hours. This result was rather surprising as no reactions were noticed at slightly lower concentrations and the chemically similar metacresol caused no fatalities at a dose of 0.3 cc. per pound of body weight and gave no indications of injury to the dog. It may be shown, in further tests, that the drug may be less toxic than indicated by the fatalities noted above.

The compound 2-tert.-butyl-4-bromophenol, given in doses from 0.05 to 0.3 cc. per pound of body weight, expelled 100 per cent (69) of the ascarids present in dogs. Its efficacy in removing hookworms appears to be variable since 4.7 to 100 per cent removal was obtained. The dog expelling the smallest percentage of hookworms, 2 of 42 worms present, received 0.05 cc. per pound of body weight. In another instance, at the same dose, 72 per cent of 43 hookworms were removed. However, of the total 244 hookworms (*Ancylostoma caninum*) present in 7 dogs, 80 per cent were evacuated following the administration of this phenol. There was no evidence of any pathologic change in the test animals and no vomiting occurred after administration of the drug. This compound appears to be worthy of further study.

The compound 4-tert.-butyl-2-chlorophenol also appeared to have definite ascaricidal value. However, 2 of the 6 dogs vomited following treatment. Enzie[8] also tested this compound on 2 dogs and found it to be effective against ascarids and hookworms. At doses varying from 0.1 to 0.2 cc. per pound of body weight of 2-tert.-butyl-4-chlorophenol, 50 per cent of the 12 ascarids present in the test animals were removed. It was 100 per cent effective in the removal of tapeworms (Dipylidium and Taenia) in one test. In a second experiment, however, only the segments were expelled, leaving 6 Taenia heads. Little or no action on hookworms was noted. The compounds 4-tert.-amyl-2-chlorophenol and phenylethylphenol removed 81 and 87.5 per cent, respectively, of the ascarids present but neither appeared to have value as a treatment for hookworms. The remaining disubstituted phenolic compounds appear to have little value as anthelmintics, and, at the same time, have other disturbing properties which appear to cause vomiting and constipation. Nitrothiocyanophenol appears to be extremely poisonous to dogs since it killed both test animals within a few hours.

Among the trisubstituted phenols investigated, only 3,5-dimethyl-4-chlorophenol appears to be worthy of further investigation (table 4). This compound expelled 97.5 per cent of 40 ascarids and 53 per cent of the 349 hookworms present. It was found to be nonirritating to the mucous membrane of the mouth of the investigator and appeared to have only a slight phenolic taste. This seems to indicate that the material should be especially safe for administration to animals and thus particularly worthy of continued investigation. The four remaining trisubstituted phenols gave little evidence of value as anthelmintics. One tetra- and one pentasubstituted phenol were included in these studies and were found to be of little value (table 5).

DISCUSSION

When these investigations were first undertaken, it was hoped that some definite evidence of the relationship of chemical structure to anthelmintic action would be established. It was found that certain mono-, di-, and trisubstituted phenols were effective in removing ascarids and of possible value in the removal of hookworms and tapeworms. Other closely related materials were ineffective against these same parasites when administered under the same conditions. None of these were effective in removing whipworms, which may be due to the location of this parasite and to the low volatility of the compounds under investigation. While there was a tendency for some of these phenols to cause vomiting in dogs, on the whole, they appeared to be nontoxic with one or two notable exceptions. The relationship between position, nature, and molecular weight of the substituent group on the phenolic nucleus and anthelmintic efficacy remains obscure. Undoubtedly such relationships exist, but, to definitely establish these effects, more extensive and elaborate tests must be undertaken. It is doubtful that such tests are now justified.

Several of the compounds investigated appear to be worthy of further study. While we cannot explain, chemically or pharmacologically, the cause of their relatively more valuable characteristics, certain of them might be found to be useful for the control of the common parasites in dogs and other animals. Further investigations are proceeding with these selected compounds, and it is the hope of the writer to

TABLE 3—Anthelmintic Value of Disubstituted Phenol Compounds

Compound	Exp. (No.)	No. dogs used	Dose per lb. body wt.	Post-treatment fecal exam.	Autopsy findings	Efficiency (%)	Remarks
6-Tert.-butyl-m-cresol	9	2	0.1 cc.	6 ascarids 21 hookworms	5 hookworms	100 81.0	No reaction observed.
	10	2	0.2 cc.	30 ascarids	Negative	100	3 ascarids expelled preceding treatment. 28 ascarids expelled within 4 hours after receiving drug, the remainder within 24 hours. No reaction observed.
	15	2	0.3 cc.	16 ascarids	1 ascarid (11 mm. long) 4 ascarids (20 mm. long) 10 hookworms	100 0	Defecated 30 minutes after receiving drug, frothed at mouth. Vomited 3 hours after receiving drug. Feces had strong odor of drug. The ascarids found at autopsy, conducted 14 days after receiving drug, were probably not in intestine at time drug was administered.
2-Tert.-butyl-p-cresol	291	2	0.1 Gm.	2 ascarids 7 hookworms	15 hookworms 12 whipworms	100 36.8 0	Defecated within 2½ hours.
	303	2	0.2 Gm.	62 ascarids	9 whipworms	100	No reaction noted.
				13 Dipylidium 27 Taenia		100 100	
	268	2	0.3 Gm.	.....	.....		No symptoms for 3 hours after treatment. Both dogs died within 17 hours after receiving drug. Drug highly toxic at 0.3 Gm. per pound of body weight.
4-Tert.-amyl-o-cresol	16	2	0.1 cc.	5 ascarids	3 ascarids	62.5	Appears to be constipating.
	84	2	0.1 cc.	9 ascarids 1 hookworm	4 ascarids 6 hookworms Many Dipylidium	69.0 14.0 0	No reaction noted.
Thiocyano-o-cresol	123	2	0.1 Gm.	9 ascarids	6 ascarids 24 whipworms	60.0 0	1 ascarid expelled preceding treatment. Vomited 2 hours after receiving drug.
4-Tert.-amyl-2-chloro-phenol	74	2	0.1 cc.	13 ascarids	4 hookworms	100 0	1 ascarid expelled preceding treatment. A mild compound, apparently had no effect on dog.
	83	2	0.1 cc.	1 ascarid	16 whipworms 1 Dipylidium 7 Taenia	100 0 0 0	Slight vomiting in 2 dogs.
	130	2	0.1 cc.	3 ascarids 2 hookworms	4 ascarids 1 hookworm 3 Taenia	43.0 66.0 0	2 ascarids expelled preceding treatment. No reaction observed.
4-Tert.-butyl-2-chloro-phenol	50	2	0.1 cc.	9 ascarids	6 ascarids	60.0	1 ascarid expelled preceding treatment.
	132	2	0.1 cc.	53 ascarids	1 hookworm	100 0	10 ascarids expelled preceding treatment. Two dogs vomited following treatment.
2-Tert.-butyl-4-chloro-phenol	278	2	0.1 cc.	5 ascarids 1 whipworm 32 Dipylidium 5 Taenia	22 whipworms	100 4.0 100 100	No reaction noted.
	282	1	0.2 cc.	Negative	7 hookworms	0	No reaction noted.
	322	2	0.2 cc.	1 ascarid 1 whipworm	25 whipworms 6 ascarids	100 3.8 0	No reaction noted.
	324	2	0.1 cc.	Many Taenia segments	22+ whipworms 6 Taenia heads	0 0	1 ascarid expelled preceding treatment.

TABLE 3 (continued)—Anthelmintic Value of Disubstituted Phenol Compounds

Compound	Exp. (No.)	No. dogs used	Dose per lb. body wt.	Post-treatment fecal exam.	Autopsy findings	Efficiency (%)	Remarks
2-Tert.-butyl-4-bromo-phenol	280	2	0.1 cc.	2 hookworms 3 whipworms	14 hookworms 45 whipworms	12.5 6.0	No reaction noted.
	285	3	0.3 cc.	36 ascarids 19 hookworms 2 whipworms	71 whipworms	100 100 2.0	2 ascarids expelled preceding treatment. May have slight laxative effect.
	287	2	0.2 cc.	3 ascarids 135 hookworms	3 hookworms 24 whipworms	100 97.8 0	May have slight laxative effect.
	299	2	0.3 cc.	11 ascarids	1 hookworm	100 0	May have slight laxative effect.
	302	2	0.05 cc.	19 ascarids 33 hookworms	52 hookworms	100 38.8	No apparent reaction.
Phenylethylphenol (Beta)	217	2	0.1 Gm.	8 ascarids 2 hookworms	4 ascarids 114 hookworms	66.6 1.8	3 ascarids expelled preceding treatment. No reaction noted.
	289	2	0.1 Gm.	27 ascarids	3 whipworms	100 0	No reaction noted.
2-Methoxy-4-methyl-phenol	174	2	0.1 cc.	1 ascarid	1 ascarid 5 Dipylidium	0 0	2 ascarids expelled preceding treatment. No reaction observed.
	320	3	0.1 cc.	4 ascarids	11 ascarids	26.6	No reaction observed.
	326	2	0.1 cc.	1 ascarid	2 ascarids 37 hookworms	0 0	2 ascarids expelled preceding treatment. Defecated within 15 minutes after receiving drug.
2,4-Dimethylphenol	126	2	0.1 cc.	2 hookworms	4 hookworms 7 whipworms many Taenia	33.0 0 0	Vomited 3 hours after receiving drug. Unsatisfactory, due to its emetic effect.
	334	2	0.1 cc.	2 ascarids	Numerous whipworms many Taenia	100 0 0	Vomited very little 1 hour after receiving drug, became progressively worse; lost appetite
Diamylphenol	288	2	0.1 cc.	8 ascarids	2 ascarids 42 whipworms	80.0 0	4 ascarids expelled preceding treatment. Passed stool in 2 hours. Questionable value.
	321	2	0.1 cc.	4 ascarids	2 ascarids	50.0	Passed stool within 2 hours after receiving drug.
	325	2	0.1 cc.	2 ascarids	44 ascarids 15 hookworms 14 whipworms	0 0 0	12 ascarids expelled preceding treatment. No reaction noted.
	327	2	0.1 cc.	2 ascarids 25 hookworms	243 hookworms 1 whipworm few Dipylidium few Taenia	100 9+ 0 0 0	No reaction noted.
Nitrothiocyanophenol	17	1	0.1 Gm.	.....	many ascarids and hookworms		Both dogs died within 3 hours after receiving compound. Seemed to show symptoms of asphyxia. Highly toxic.
	17	1	0.05 Gm.	.....	moderate ascarids		

TABLE 4—Anthelmintic Value of Trisubstituted Phenol Compounds

Compound	Exp. (No.)	No. dogs used	Dose per lb. body wt.	Post-treatment fecal exam.	Autopsy findings	Efficiency (%)	Remarks
2,6-Ditert.-butyl-p-cresol	26	2	0.1 Gm.	3 ascarids	86 ascarids	3.0	No reaction noted.
4-Tert.-amyl-2,6-dichlorophenol	67	2	0.1 cc.	negative	4 ascarids 1 whipworm	0 0	No reaction noted.
2,6-Ditert.-butyl-4-chlorophenol	279	2	0.1 Gm.	negative	12 whipworms 28 Taenia	0 0	2 ascarids expelled preceding treatment. No conclusions, bad state of health. Drug seemed to pass through animal unchanged, insoluble.
	329	2	0.1 Gm.	1 ascarid 26 hookworms	1 ascarid 216 hookworms 1 whipworm	50.0 10+ 0	Compound seemed insoluble in digestive tract.
3,5-Dimethyl-4-chlorophenol	328	2	0.1 Gm.	3 ascarids	1 ascarid 2 hookworms 8 whipworms	75.0 0 0	No reaction noted.
	330	2	0.1 Gm.	23 ascarids	negative	100	1 ascarid expelled preceding treatment. No reaction noted.
	331	2	0.2 Gm.	5 ascarids	negative	100	No reaction noted.
	335	2	0.1 Gm.	8 ascarids 210 hookworms	182 hookworms	100 53.6	8 hookworms, 1 whipworm expelled preceding treatment. One of the dogs passed stool within 1 hour following drug.
				16 whipworms few Taenia	several hundred whipworms	0 100	
4-Thiocyano-3-methyl-nitrophenol	82	2	0.1 Gm.	.....	.....	.....	Both animals were in generalized tonic convulsions 4 hours after receiving drug and died within 18 hours after receiving compound.

TABLE 5—Anthelmintic Value of Tetra-and Pentasubstituted Phenol Compounds

Compound	Exp. (No.)	No. dogs used	Dose per lb. body wt.	Post-treatment fecal exam.	Autopsy findings	Efficiency (%)	Remarks
2,4-Dimethyl-(3,5)6-diethylphenol	294	2	0.1 cc.	11 ascarids	1 ascarid	91.6	No reaction noted.
	323	1	0.1 cc.	2 ascarids 2 hookworms	4 ascarids 10 hookworms	33+ 16.0	No reaction noted.
2,4-Dimethyl-3,5,6-triethylphenol	293	2	0.1 cc.	3 ascarids	22 ascarids 10 hookworms	12.0 0	No reaction noted.

be able to report the results in the near future.

CONCLUSIONS

1) Twenty-six substituted phenols were studied for anthelmintic properties through tests on dogs.

2) Ten of these phenol compounds were shown to have value in removing ascarids, hookworms, and tapeworms from the test animals, but none were effective against whipworms.

3) The mixed-2-amylphenol, 2-tert.-butyl-4-bromophenol, 3,5-dimethyl-4-chlorophenol, and the technical grade of 6-tert.-butyl-m-cresol were found to have sufficient promise to warrant further study on dogs and other animals for the treatment and control of ascarids, tapeworms, hookworms, and other parasites.

4) No definite relationship between the chemical structure and anthelmintic action could be established in these investigations with this series of phenolic compounds.

References

[1]Bozzolo, C.: Di un'altra sostanza attiva contro l'anchilostoma Dubini. Osservatore, Torino, *17*, (1881):1-2.

[2]Lamson, P. D., Ward, C. B., and Brown, H. W.: An Effective Ascaricide-Hexylresorcinol. Proc. Soc. Exptl. Biol. and Med., *27*, (1930):1017-1020.

[3]Lamson, P. D., and Ward, C. B.: The Chemotherapy of Helminth Infestation. J. Parasitol., *18*, (1932):173-199.

[4]Lamson, P. D., Brown, H. W., and Ward, C. B.: Anthelmintic Studies of Alkylhydroxybenzenes. I. Alkylpolyhydroxybenzenes. J. Pharmacol. and Exptl. Therap., *53*, (1935):198-217.

[5]Lamson, P. D., Brown, H. W., Stoughton, R. W., Harwood, P. D., Baltzly, R. and Bass, A.: Anthelmintic Studies on Alkylhydroxybenzenes. IV. Isomerism in Polyalkylphenols. J. Pharmacol. and Exptl. Therap., *55*, (1935):234-238.

[6]Lamson, P. D., Brown, H. W., Stoughton, R. W., Harwood, P. D., Baltzly, R., and Bass, A. D.: Anthelmintic Studies on Alkylhydroxybenzenes. V. Phenols with Other than Normal Alkyl Side Chains. J. Pharmacol. and Exptl. Therap., *59*, (1935):239-249.

[7]Enzie, F. D.: The Anthelmintic Action of "Butylphen" in Dogs. Proc. Helminthol. Soc. Washington, *11*, (1944):55-58.

[8]Enzie, F. D.: Anthelmintic Studies with Some Thymol-Like Compounds. Proc. Helminthol. Soc. Washington, *12*, (1945):19-24.

The Influence of Atmospheric Gases on the Multiplication of Brucella

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IT HAS not been possible, until recently, to obtain quickly a large, live cell population of members of the genus Brucella in large volumes of liquid culture medium. This was accomplished to a limited degree in another laboratory by keeping fortified tryptose liquid medium aërated during the growth of *Brucella suis*.[1]

One of the troublesome problems which develops as a result of growing Brucella in a liquid medium is the marked tendency of the bacterial cells to undergo dissociation during growth. It is most essential that changes in the growth phase of organisms be prevented in liquid culture mediums, if the cells are to be used as antigens or vaccines, or if cell metabolism studies are to be undertaken.

The problem of producing large, live cell populations of the species of Brucella in any pure growth phase in a simple liquid culture medium has been solved through a study of the gaseous requirements and a method of making available the requirements by agitation of the medium during the growth period.

EXPERIMENTAL PROCEDURES AND RESULTS

Difco tryptose was selected as the peptone base for the liquid medium, since it has been employed in this laboratory for more than ten years for growing Brucella and since practicality rather than definition was a prime requisite of the medium sought.

In the preliminary studies, the first basal medium used contained the following constituents:

Tryptose	1.0 Gm.
Glucose	1.0 Gm.
NaCl	0.5 Gm.
Distilled water	100.0 ml.

The pH of the medium was adjusted to 6.7 with phosphoric acid. The medium was distributed in 15-ml. amounts into pyrex tubes 8 in. by 1 in., plugged with absorbent cotton, and autoclaved at 115 C. for fifteen minutes.

After inoculation with a standard inoculum (5×10^6 viable cells per milliliter), the medium was incubated at 37 C. for seven days on a push-pull type of shaker, operating at a rate of 75 strokes per minute. The average maximum viable count of *Br. suis* in the basal medium in the stated conditions was 18×10^8 cells per milliliter.

The addition of thiamine hydrochloride to the basal medium to a final concentration of 0.5 mg. per 100 ml. resulted in a stimulation of the growth of all species of Brucella. Counts of $35 \pm 5 \times 10^8$ viable cells per milliliter were consistently obtained with *Br. suis*. The addition of a number of other vitamins, amino acids, and minerals which have been reported as essential or stimulatory for growth of Brucella[1-7] failed to increase the viable cell count to an appreciable degree.

The basal medium just described, containing 0.5 mg. per 100 ml. of thiamine hydrochloride, when used in large volumes, did not support growth comparable to that obtained in test tubes. Attempts to obtain larger numbers of bacteria resulted in a study of the influence of atmospheric gases on growth.

Preliminary investigations indicated that the maximum growth of Brucella cells in the fortified tryptose liquid medium was obtained only when there was a continuous supply of oxygen in the space above the medium and constant vigorous agitation of the liquid. When the demand of the multiplying cells for oxygen was met, it was found that the medium, which was more than adequate under former culture conditions, did not contain sufficient nutrients to produce maximum populations. Therefore, the concentrations of the constituents in the medium were reconsidered.

A series of mediums was prepared which contained various concentrations of tryptose and glucose as well as thiamine and sodium chloride. Two hundred and fifty milliliters of each medium were placed in 1-liter bottles fitted with rubber stoppers through which passed two pieces of glass tubing. One of these served as the delivery tube for the gas or gases under study and the other the gas outlet. The gas delivery tube extended into the culture container to a point about 2.5 cm. above the surface of the liquid. Glass floats were suspended in the liquid to intensify the movement of the liquid and the distribution of gases into the medium. The bottles of mediums were autoclaved at 115 C. for fifteen minutes and, after inoculation, were incubated at 37 C. on a push-pull type of shaker.* The rate of gas

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*Since the completion of this study, more efficient agitation of the medium has been obtained

flow was measured by a rotameter. Bottles containing like amounts of each medium and fitted with cotton plugs were inoculated and placed on the shaker as controls.

A single smooth strain of each species of Brucella was used in all further studies. A suspension of 24-hour growth from liver agar slants was used for inoculum. In most of the experiments, the medium was seeded with 10^6 cells per milliliter. During incubation, samples were removed at 24-hour intervals. The viable cell count was determined by plating suitable dilutions of the cultures on tryptose agar. The total cell count was measured turbidimetrically in a photelometer from a standard curve established by direct cell counts. Residual glucose was determined colorimetrically by a modified Benedict's procedure[10] and the pH was measured electrometrically. The growth phase of each culture was ascertained by examination of four-day-old colonies under a low power stereoscopic microscope.[11]

Effects of Air and Oxygen.—As more optimum conditions for multiplication and metabolism of the organisms were established, the concentrations of the constituents of the medium were increased to the minimum amount which produced maximum response. An initial concentration of 20 mg. of glucose per milliliter was needed to assure a slight excess of the carbohydrate in the medium after ninety-six hours' incubation in an atmosphere of pure oxygen. There was no additional increase in cell counts when thiamine hydrochloride was present in amounts greater than 0.5 mg. per 100 ml.

The effects of maintaining different atmospheric conditions on the multiplication of *Br. suis* in each of three mediums are illustrated by the growth curves in figure 1. The atmospheric conditions that were maintained during the incubation period of ninety-six hours were (A) stagnant air, the bottles being fitted

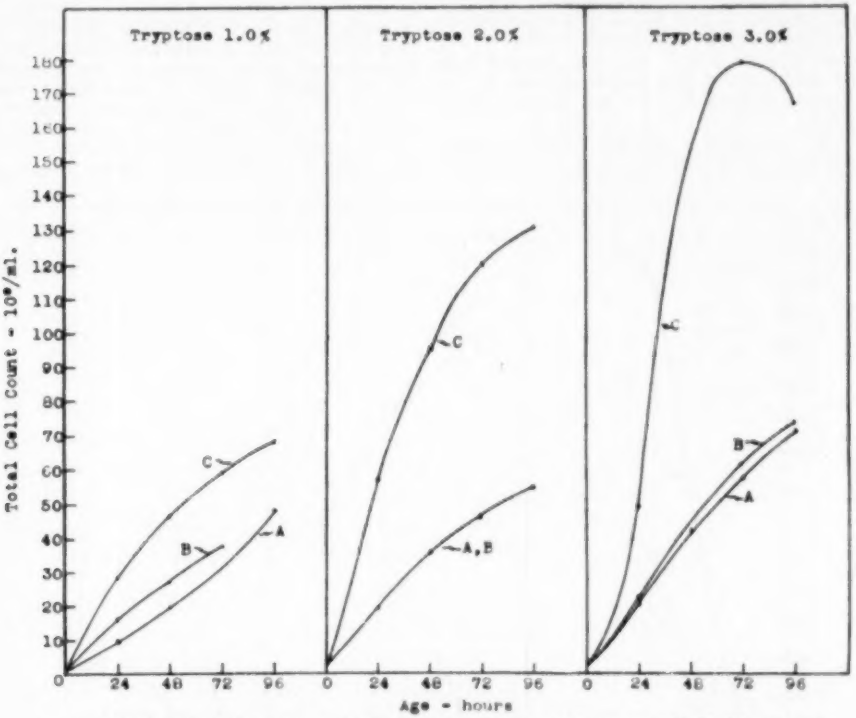

Fig. 1—Multiplication of *Brucella suis*. Medium: tryptose; glucose, 2.0 per cent; NaCl, 0.5 per cent; thiamine-HCl, 0.5 mg. per 100 ml. of medium. Atmosphere: A, stagnant air; B, flowing air; C, flowing oxygen.

by means of a variable angle shaker with hydraulic transmission without the aid of glass floats in the culture bottle.

with cotton plugs, (B) a constant flow (85 ml. per minute) of filtered air, and (C) a constant flow (45 ml. per minute) of pure oxygen. The three mediums differed only in the concentration of tryptose. The final cell populations of *Br. suis* and *Brucella melitensis* were larger in all mediums exposed to flowing oxygen than in those exposed to flowing or stagnant air. The acceleration of growth and metabolism in pure oxygen was enhanced as the concentration of tryptose was increased. Results were not significantly different when the rate of flow of oxygen was varied from 15 to 105 ml. per minute.

The growth response of *Brucella abortus*, under the conditions just mentioned, appeared more dependent than the other two species on the concentration of tryptose. Only in the medium containing 3 per cent tryptose was the cell count of this species greater in the presence of pure oxygen than in the control culture.

The comparative studies, therefore, show that large cell populations of each of the three species of Brucella can be obtained in a short time in large volumes of culture medium in the presence of pure oxygen and without the appearance of dissociated phases, if the constituents of the medium are used in the following concentrations:

Tryptose	3.0 Gm.
Glucose	2.0 Gm.
Thiamine·HCL	0.5 mg.
NaCl	0.5 Gm.
Distilled water	100.0 ml.

The productive capacity of this medium is illustrated by the curves in figure 2. These show the total and viable cell counts of each of the three species of Brucella.

Effect of Nitrogen and Carbon Dioxide.—Consideration has been given to the effect on cell multiplication of other gases such as pure nitrogen and carbon dioxide and combinations of these with oxygen. It was found that growth of all species in the medium described above was suppressed in an atmosphere of pure nitrogen or pure carbon dioxide. When the inoculated medium was exposed to a mix-

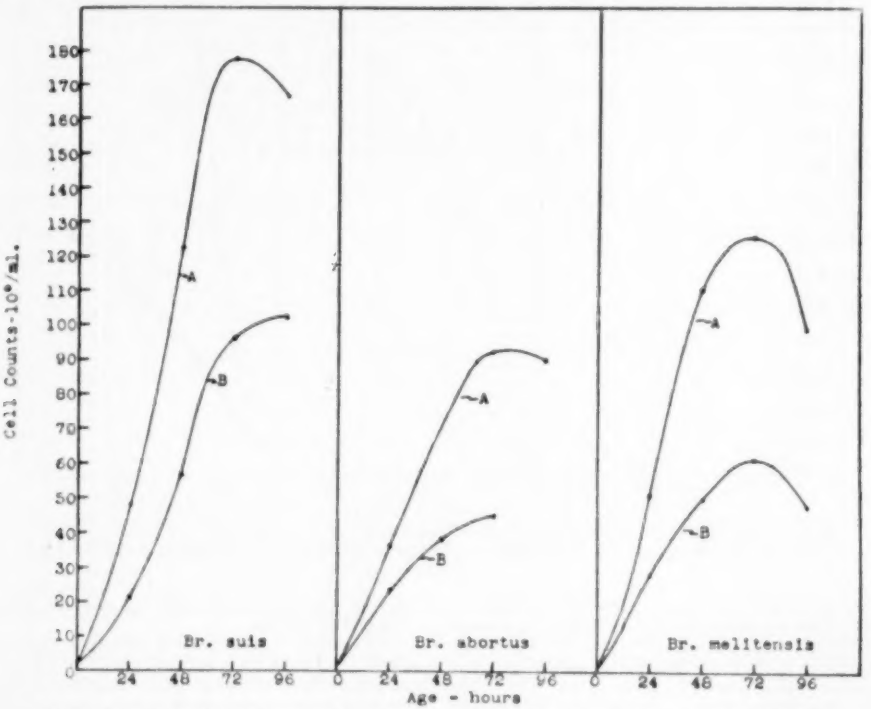

Fig. 2—Total and viable cell counts under optimum conditions. Cell counts: A, total; B, viable. Medium: tryptose, 3.0 per cent; glucose, 2.0 per cent; NaCl, 0.5 per cent; thiamine·HCl, 0.5 mg. per 100 ml. of medium. Atmosphere: flowing oxygen (45 ml./min.).

ture of equal amounts of flowing nitrogen and oxygen, all species of Brucella grew at about the same rate and reached a maximum cell count comparable to that obtained in flowing air.

In a study of the effect of mixtures of carbon dioxide and oxygen, the individual response of each of the species suggests that their oxygen requirements are not equivalent. The flow of carbon dioxide was maintained constant to each culture while the flow of oxygen was varied so that the ratios of oxygen to carbon dioxide were approximately 2 : 1, 9 : 1, and 18 : 1. The partial pressure of the oxygen in the atmosphere governed the amount which was dissolved in the medium and available to the bacterial cells. The data in figure 3 show that the cell count of *Br. suis* was directly related to the amount of oxygen available and was higher in pure oxygen than in combinations of oxygen and carbon dioxide. The ratio of 18 : 1 was as satisfactory as pure oxygen for *Br. melitensis*. The multiplication of *Br. abortus* in any of the mixtures of these gases was equal to that in pure oxygen.

DISCUSSION

Large populations of the species of Brucella have been obtained in liquid mediums only under conditions which make available to the cells large amounts of oxygen. A continuous stream of pure oxygen gas supplied to a vigorously shaken culture for the duration of the incubation period accelerated the rate of multiplication.

Winslow *et al.*[12] were among the first to

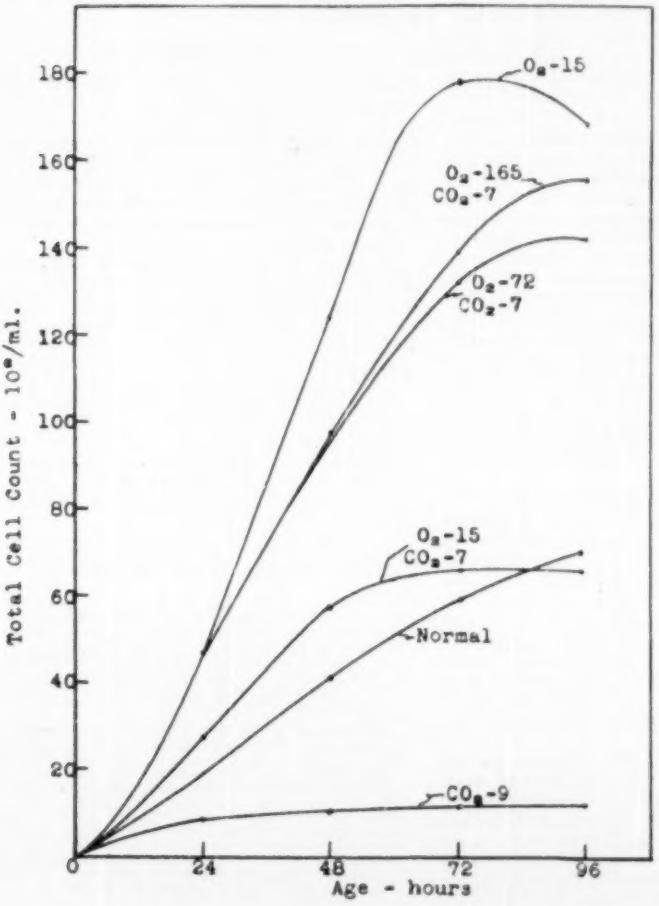

Fig. 3 — Multiplication of *Brucella suis* in atmospheres of oxygen and carbon dioxide. O_2, CO_2—ml./min. Medium: tryptose, 3.0 per cent; glucose, 2.0 per cent; NaCl, 0.5 per cent; thiamine-HCl, 0.5 mg. per 100 ml. of medium.

show that aëration resulted in a great increase in the population of facultative anaërobes. The increase was attributed to removal of toxic products of growth and to enhanced oxygenation. Rahn and Richardson[13,14] investigated the oxygen demands of various species of organisms and found that the rates of multiplication in 1 per cent peptone were the same until the oxygen was almost completely exhausted but that the tolerance of aërobic organisms for anaërobic conditions varied with different groups. When oxygen in the medium was exhausted, the multiplication of most facultative anaërobes was constant only at the surface. The limiting factor for large populations was the availability of oxygen. Levine[15] observed that the growth of spores and vegetative cells of *Bacillus subtilis* was not reduced by an atmosphere of 100 per cent oxygen. The minimum amount that would support normal growth was 20 per cent. Roby[8] noted a considerable increase in the multiplication of Brucella cells when the culture medium was aërated by agitation. Similar results from studies of *Br. suis* in which the culture medium was aërated have been reported.[16,17,18,19]

It was found, in the present study, that a medium which was adequate for Brucella when maintained in normal atmospheric conditions did not contain a sufficient concentration of nutrients to support maximum cell populations in an atmosphere of oxygen. Maximum cell counts of each of the species of Brucella were obtained when the medium contained 3 per cent tryptose, 2 per cent glucose, 0.5 mg. per 100 ml. thiamine hydrochloride, and 0.5 per cent sodium chloride. Dissociated growth phases of Brucella were not observed in the liquid medium that was shaken constantly during the growth period.

Other gases and mixtures of gases were used to determine if the effect observed with pure oxygen was a specific reaction or if the results were due to the removal of toxic metabolic products from the medium. Pure nitrogen flowing into bottles of inoculated culture medium suppressed the growth of all species. Neither a mixture of equal volumes of nitrogen and oxygen nor a continuous flow of air resulted in a total cell count that was higher than that which developed in bottles of medium stoppered with cotton plugs.

Although an atmosphere of pure carbon dioxide inhibited growth, the rate of multiplication of Brucella cells in a medium exposed to a mixture of oxygen and carbon dioxide in a ratio of 18:1 was equal to or approached the rate obtained when pure oxygen was used. *Brucella abortus* exhibited a much greater tolerance for high concentrations of carbon dioxide than *Br. suis* or *Br. melitensis*. In view of the requirements of *Br. abortus* for carbon dioxide for primary isolation from pathologic material,[20] this finding was not unexpected.

The data demonstrate that there is a demand for large amounts of oxygen by the species of Brucella. The atmosphere to which the culture medium is exposed must approach a concentration of 100 per cent oxygen, and the liquid must be constantly agitated in order to make available in the medium a sufficient amount of this gas.

Cell counts of *Br. abortus* did not equal those of *Br. melitensis* or *Br. suis* under any of the conditions studied. It is possible that a more rapid multiplication of *Br. abortus* could be obtained by some additional nutritional factor, a different atmosphere, or both.

SUMMARY

Results from the study of the influence of atmospheric gases on the multiplication of the three species of Brucella in liquid culture mediums show conclusively that controlled atmospheres are equally as important as the medium constituents. Rates of multiplication and cell counts are greatly increased when these organisms are grown in an atmosphere of pure oxygen.

A practical liquid medium is described which, when exposed to pure oxygen and constantly agitated, produces large live cell populations of a pure growth phase of the Brucella.

References

[1]McCullough, W. G., Mills, R. C., Herbst, E. J., and Roessler, W. G.: Studies on the Nutritional Requirements of *Brucella Suis*. J. Bact., *53*, (1947):5-15.

[2]Kerby, G. P.: Nicotinic Acid and Thiamin Hydrochloride as Growth-Promoting Factors for Brucella. J. Bact., *37*, (1939):495-499.

[3]Koser, S. A., Breslove, B. B., and Dorfman, A.: Accessory Growth Factor Requirements of Some Representatives of the Brucella Group. J. Infect. Dis., *69*, (1941):114-124.

[4]Koser, S. A., and Wright, Marjorie H.: Further Experiments on Accessory Growth Factor Requirements of the Brucella Group. J. Infect. Dis., *71*, (1942):86-88.

[5]McCullough N. B., and Dick, L. A.: Physiological Studies of Brucella. I. Quantitative Accessory Growth Factor Requirements of Certain Strains of Brucella. J. Infect. Dis., *71*, (1942):193-197.

[6]McCullough, N. B., and Dick, L. A.: Physiological Studies of Brucella. II. Accessory Growth Factor Requirements of Recently Isolated Strains of *Brucella Abortus*. J. Infect. Dis., *71*, (1942): 198-200.

[7]McCullough, N. B., and Dick, L. A.: Growth of Brucella on a Simple Chemically Defined Medium. Proc. Soc. Exptl. Biol. and Med., *52*, (1943):310-311.

[8]Roby, T. O.: A Study of the Growth Requirements of the Genus Brucella. Thesis, Michigan State College, East Lansing, Mich., 1944.

[9]Gerhardt, Philipp, and Wilson, J. B.: The Nutrition of Brucellae: Growth in Simple Chemically Defined Media. J. Bact., *56*, (1948):17-24.

[10]Benedict, S. R.: Determination of Blood Sugar, II. J. Biol. Chem., *76*, (1928):457-470.

[11]Huddleson, I. F.: The Mucoid Phases of the Genus Brucella. Am. J. Vet. Res., 7, (1946):5-16.

[17]Winslow, C. E. A., Walker, H. H., and Sutermeister, M.: The Influence of Aeration and Sodium Chloride upon the Growth Curve of Bacteria in Various Media. J. Bact., *24*, (1932):185-208.

[18]Rahn, Otto, and Richardson, G. L.: Oxygen Demand and Oxygen Supply. J. Bact., *41*, (1940): 225-249.

[19]Rahn, Otto, and Richardson, G. L.: Oxygen Demand and Oxygen Supply. J. Bact., *44*, (1942): 321-332.

[20]Levine, P. P.: The Effect of Atmospheres of Hydrogen, Carbon Dioxide and Oxygen Respectively and of Mixtures of these Gases on the Growth of *Bacillus Subtilis*. J. Bact.,*31*, (1936): 151-160.

[21]Gee, Lynn L., and Gerhardt, Philipp: *Brucella Suis* in Aerated Broth Culture. II. Aeration Studies. J. Bact., *52*, (1946):279-281.

[22]Gerhardt, Philipp: *Brucella Suis* in Aerated Broth Culture. III. Continuous Culture Studies. J. Bact., *52*, (1946):283-292.

[23]Gerhardt, Philipp, and Gee, Lynn L.: *Brucella Suis* in Aerated Broth Culture. I. Preliminary Studies on Growth Assays, Inoculum, and Growth Characteristics in an Improved Medium. J. Bact., *52*, (1946):261-269.

[24]Glassman, H. N., and Elberg, S.: The Growth of Brucella in Aerated Liquid Cultures. J. Bact., *52*, (1946):423-430.

[25]Huddleson, I. F.: The Importance of an Increased Carbon Dioxide Tension in Growing *Bacterium Abortus* (Bang). The Cornell Vet., *11*, (1921):210-215.

The Influence of Oxygen on the Metabolic Activities of Brucella

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IN VIEW OF the demonstration that the accelerating effect of oxygen and agitation on multiplication of Brucella was accompanied by the decomposition of large amounts of glucose,[1] it was considered of academic interest to study some phases of the metabolic activities of the organisms grown under the conditions which were optimum for the production of large bacterial populations. The amounts of glucose decomposed under different conditions were determined and the pH changes in various mediums were observed. Experiments were designed to study the role of thiamine in carbohydrate utilization and the fate of glucose and peptone by the determination of certain products of aërobic oxidation. Pyruvic acid and lactic acid were substituted for glucose to determine utilization of these substrates.

EXPERIMENTAL PROCEDURES AND RESULTS

The medium used in a majority of the experiments was:

Tryptose	3.0 Gm.
Glucose	2.0 Gm.
Thiamine · HCl	0.5 mg.
NaCl	0.5 Gm.
Distilled water	100.0 ml.

Variations in concentrations and substitutions in the above formula are noted in discussions of the specific experiments and in the figures. The culture container, inoculum, shaking apparatus, and flow of oxygen employed in the experiments have been described previously.[1] Samples were removed at 24-hour intervals for total cell count, pH determination, and chemical analyses. The total cell count was measured turbidimetrically in a photelometer from a standard curve established by direct cell counts and pH was measured electrometrically. Glucose was determined colorimetrically by a modified Benedict's procedure[2] and pyruvic acid by the method of Clift and Cook,[3] as modified by Elliott *et al.*[4] The procedure of Friedemann and Graeser[5] was followed for the measurements of lactic acid and the method of Friedemann[6] was used for determining volatile acids. All data are representative of tests on at least two preparations of each medium.

Glucose Decomposition.—Comparisons of the amounts of glucose decomposed by the three species of Brucella were made to determine the influence of the concentration of tryptose in the medium and the effect of atmospheric conditions. Total cell counts and residual glucose were measured while cultures were growing in mediums which contained an adequate amount of glucose and 1, 2, or 3 per cent tryptose. Cultures were examined in bottles stoppered with cotton plugs and in bottles supplied with a constant flow of oxygen during a 96-hour incubation period. Calculated values of the amounts of glucose decomposed per billion cells are shown in table 1.

It may be noted that *Brucella suis* decomposed less glucose per unit number of cells than did the other two species. In both atmospheric conditions employed, each of the three species used less glucose per billion cells as the concentration of tryptose was increased. With one exception, each of the species, in any

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one of the mediums, decomposed more glucose in an atmosphere of oxygen than in stagnant air. *Brucella abortus* in 1 per cent tryptose medium decomposed more glucose in stagnant air than in an atmosphere of oxygen. The slow rate of multiplication of *Br. abortus* in this medium exposed to oxygen would indicate that the nutrients and environment were not favorable for metabolism.

Changes in pH.—The pH changes which occurred in mediums containing 1, 2, or 3 per cent tryptose without glucose and in similar mediums containing 1 or 2 per cent glucose during the growth of *Br. abortus*, *Brucella melitensis*, and *Br. suis* are shown in figures 1, 2, and 3. The mediums were continually shaken and were exposed to stagnant air or to pure oxygen.

The mediums without glucose rapidly became alkaline. In mediums containing glucose, the pH changes were, in general, characteristic of each of the species, but were influenced by the initial concentrations of both tryptose and glucose in the medium. The higher the concentration of glucose, the lower were pH values during the growth of the organisms. By increasing the concentration of tryptose, the opposite effect was observed. A distinct rise in pH was correlated with the exhaustion of the supply of glucose in the medium. Mediums inoculated with *Br. suis* remained near neutral or became alkaline during incubation, even though large amounts of glucose were decomposed.

Thiamine.—Figures 4, 5, and 6 are graphic representations of results from a study of the effects of thiamine on multiplication, glucose decomposition, and pH changes. The presence of glucose, without added thiamine, resulted in a significant increase in rates of multiplication of *Br. abortus* and *Br. melitensis*, but not of *Br. suis*, even though this species decomposed a large amount of the carbohydrate. When both glucose and thiamine were available, there was a more rapid growth and an increase in the amount of glucose decomposed by *Br. abortus* and *Br. melitensis*. The total cell count of *Br. suis* was six times greater in the

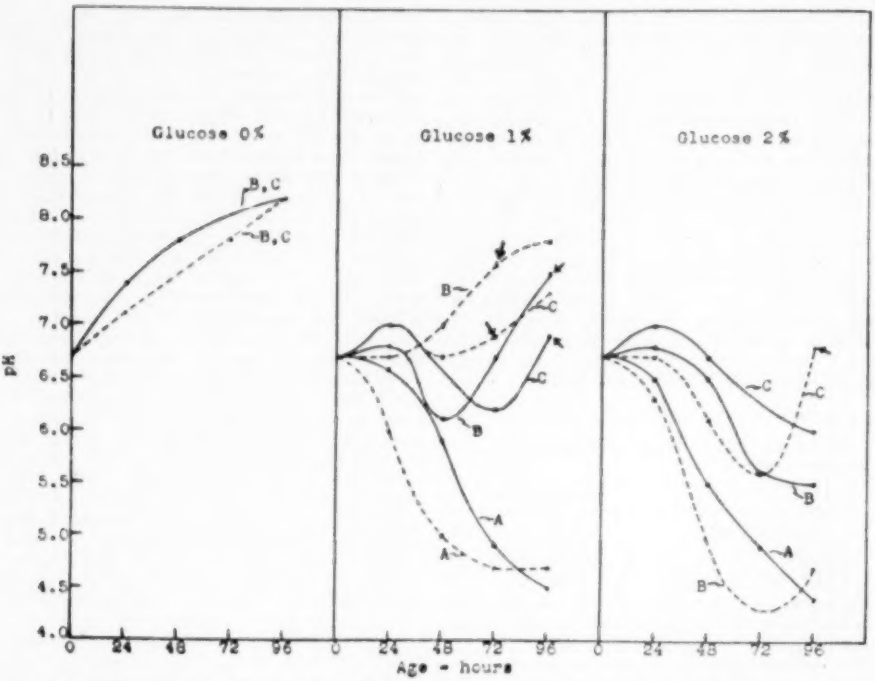

Fig. 1—Changes in pH in cultures of *Brucella abortus*. Atmosphere: ———, stagnant air; - - - -, flowing oxygen. Medium: A, tryptose, 1 per cent; B, tryptose, 2 per cent; C, tryptose, 3 per cent; glucose: NaCl, 0.5 per cent; thiamine.HCl, 0.5 mg. per 100 ml. of medium. →, residual glucose less than 1 mg. per milliliter.

medium containing thiamine than in the one without it, but the amount of glucose decomposed was only slightly higher.

Pyruvic Acid and Lactic Acid.—Numerous investigators who have studied bacterial metabolism have demonstrated that thiamine acts as a catalyst in the metabolism of pyruvic acid.[7,8,9,10] When Brucella organisms were grown in a medium without added thiamine, there was evidence of a slight accumulation of pyruvic acid, although the amounts measured in cultures of *Br. suis* were within the range of experimental error of the determination. Although only small amounts of pyruvic acid were measured in a medium without thiamine, none could be detected in a medium containing it.

Pyruvic acid was substituted for glucose in tryptose medium, and its decomposition was determined with and without added thiamine. Pyruvate was decomposed by each of the species; in ninety-six hours, 0.7 mg. per milliliter was decomposed by *Br. abortus*, 1.6 mg. per milliliter by *Br. melitensis*, and 2.9 mg. per milliliter by *Br. suis*. The addition of thiamine did not significantly alter the rates of decomposition but showed a slight stimulatory effect on the multiplication of *Br. melitensis*. The cell counts ranged from 15×10^9 cells per milliliter for *Br. abortus* to 50×10^9 cells per milliliter for *Br. suis*. The pH of the pyruvate medium increased to 8.2 to 8.5 during the growth period.

The possibility of lactic acid as an intermediate product in glucose metabolism was considered, but this acid could not be detected in cultures of any of the three species examined daily during six days of incubation. When lactic acid was substituted for glucose in tryptose medium, the quantity decomposed paralleled the cell populations. The growth of *Br. melitensis* was retarded and there was no reduction in its concentration. Growth of *Br. abortus* was slow until the third day, decomposition of lactate began simultaneously with rapid multiplication, and by the end of the 96-hour incubation period, 12 mg. of lactic acid per milliliter was decomposed. The cell

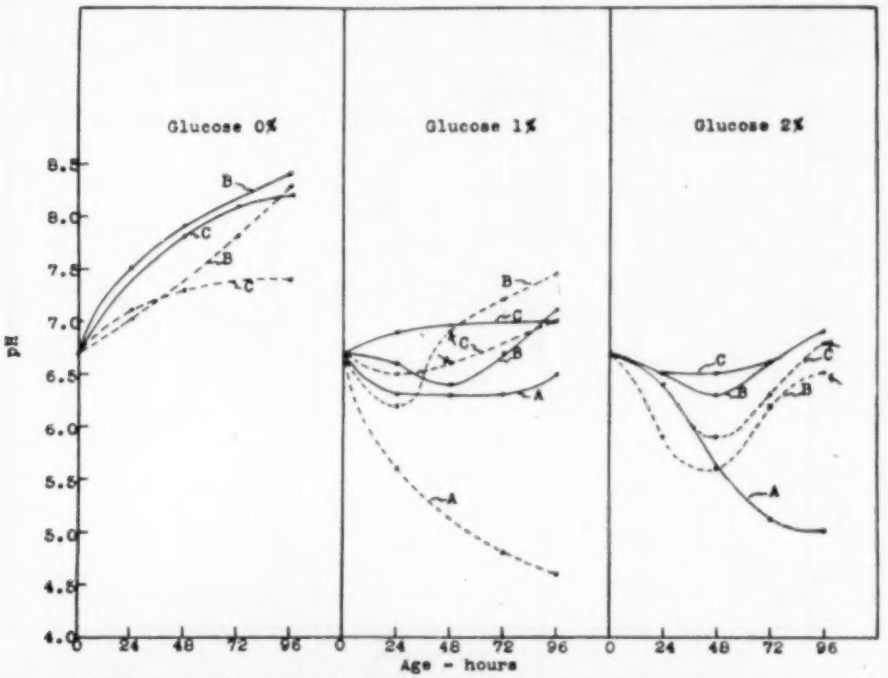

Fig. 2—Changes in pH in cultures of *Brucella melitensis*. Atmosphere: ———, stagnant air; - - - -, flowing oxygen. Medium: A, tryptose, 1 per cent; B, tryptose, 2 per cent; C, tryptose, 3 per cent; glucose; NaCl, 0.5 per cent; thiamine.HCl, 0.5 mg. per 100 ml. of medium. →, residual glucose less than 1 mg. per milliliter.

population of *Br. suis* approached that found in glucose medium, and 18 mg. of lactic acid per milliliter was decomposed in ninety-six hours.

Volatile Acids.—The presence of volatile acids as products of glucose metabolism, which could account for certain of the pH changes in the mediums, was investigated. Numerous analyses of the medium in which each of the species of Brucella was growing did not indicate the presence of a volatile acid during four days of incubation in the presence of pure oxygen.

Carbon Dioxide and Ammonia.—Since it has been shown in this study that all strains of Brucella are capable of utilizing large amounts of glucose in optimum conditions, it was interesting to observe the interrelation of peptone and carbohydrate metabolism. Quantitative measurements of carbon dioxide and ammonia were employed as estimates of carbohydrate and peptone decomposition in a tryptose medium without glucose and in one containing 2 per cent glucose.

Each of three species of Brucella was grown, as previously described, in the presence of flowing oxygen. The outlet tube from the culture container directed the exhaust gases through 1 liter of 0.01 normal sulphuric acid and, from this solution, through 1 liter of 0.3 normal potassium hydroxide. A 25-ml. aliquot was taken from the standard acid at intervals and titrated with sodium hydroxide. A 50-ml. aliquot of the potassium hydroxide was removed at the same time and, after precipitation with barium chloride, the excess potassium hydroxide was titrated with hydrochloric acid, with phenolphthalein as the indicator.

The amount of ammonia in the medium in which the bacterial cells were growing was determined by titration with 0.01 normal sulphuric acid following steam distillation of the sample into dilute boric acid. A sample of the culture medium was also aërated by a stream of alkali-washed air into 0.03 normal potassium hydroxide to measure dissolved carbon dioxide.

Determinations were made on the first four

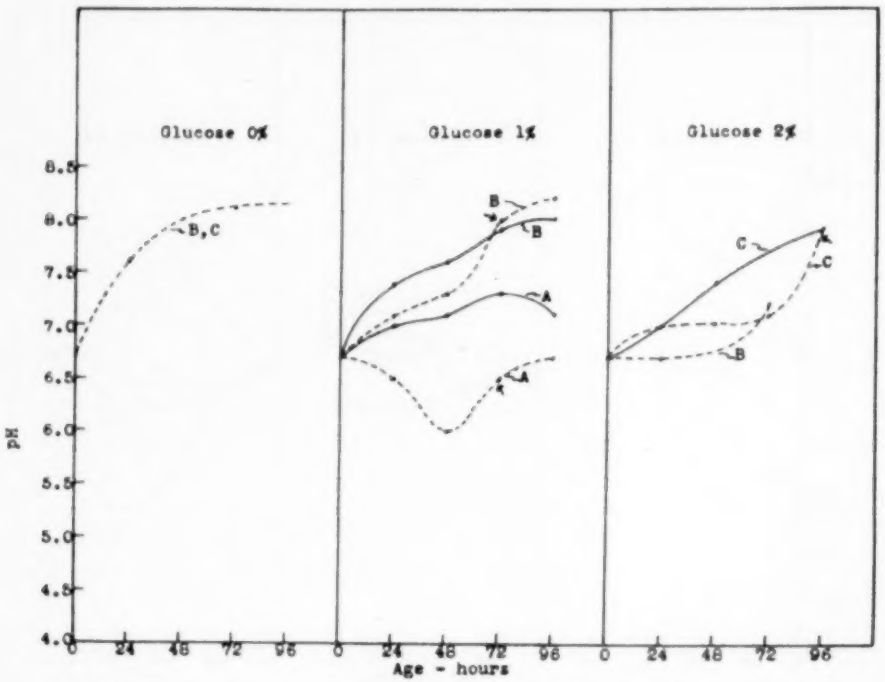

Fig. 3—Changes in pH in cultures of *Brucella suis*. Atmosphere: ———, stagnant air; - - - -, flowing oxygen. Medium: A, tryptose, 1 per cent; B, tryptose, 2 per cent; C, tryptose, 3 per cent; glucose; NaCl, 0.5 per cent; thiamine.HCl, 0.5 mg. per 100 ml. of medium. →, residual glucose less than 1 mg. per milliliter.

consecutive days and on the seventh day of incubation. Quantities of ammonia and carbon dioxide were computed as total amounts produced during each 24-hour period; the values for the seventh day were calculated as one third of that accumulated in the three previous days. Corrections were made for ammonia and carbon dioxide in uninoculated mediums. Comparisons of the rates of ammonia and carbon dioxide production by the three species of Brucella are illustrated in figure 7, and the total amounts collected during the seven-day incubation period are shown in table 2.

In the absence of glucose, the rate of metabolism of each of the species was highest during the first forty-eight hours of incubation. The total amounts of ammonia and carbon dioxide produced from tryptose by *Br. suis* were approximately two times greater than those produced by *Br. abortus* or *Br. melitensis*.

In the medium containing glucose, the carbohydrate breakdown by each of the species, as measured by the increase in carbon dioxide evolved, was greatest during the second and third days of incubation. Less carbon dioxide was produced by *Br. suis* than by the other two species. At the end of the seven-day growth period, only traces of glucose remained in the medium.

A "sparing action" of glucose on peptones was pronounced during growth of *Br. abortus* and *Br. melitensis*. No ammonia was detected until the third day in cultures of *Br. melitensis* and the fourth day in those of *Br. abortus*. In contrast, *Br. suis* attacked peptone during the first twenty-four hours; ammonia production reached a peak on the second day, and the total amount of ammonia evolved was significantly greater than that from either of the other two species.

DISCUSSION

Although glucose has often been added to various mediums to enhance the growth of Brucella, the results of previous studies of its utilization by the three species are contradictory. A classification within the genus based on glucose utilization was attempted at one time,[11] but ZoBell and Meyer[12] noted that utilization was not appreciably different in strains. McNutt and Purwin[13] reported that acid production from glucose

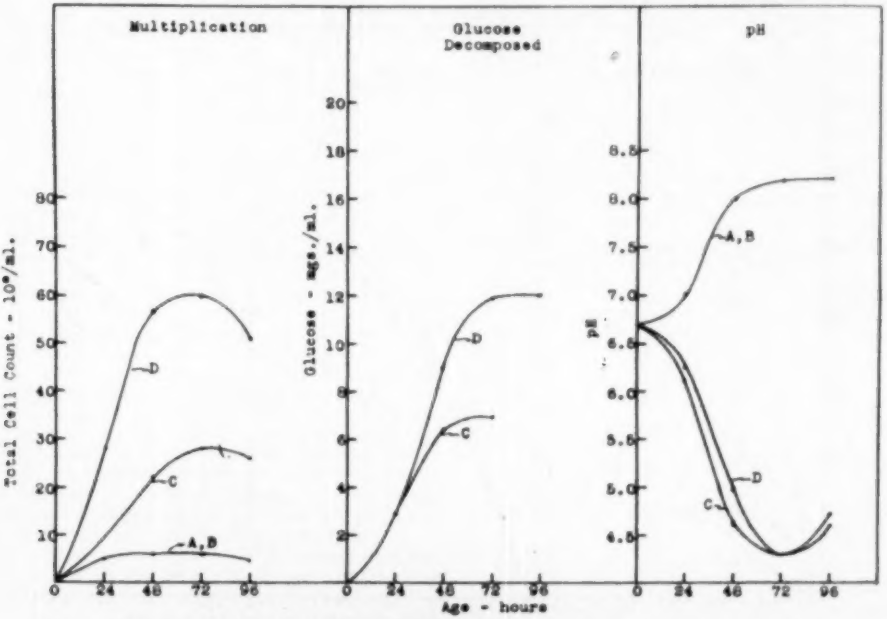

Fig. 4—Effects of thiamine and glucose on multiplication and metabolism of *Brucella abortus*. Atmosphere: flowing oxygen. Medium: A = tryptose, 2 per cent; NaCl, 0.5 per cent. B = medium A + thiamine.HCl, 0.5 mg. per 100 ml. of medium. C = medium A + glucose, 2 per cent. D = medium B + glucose, 2 per cent.

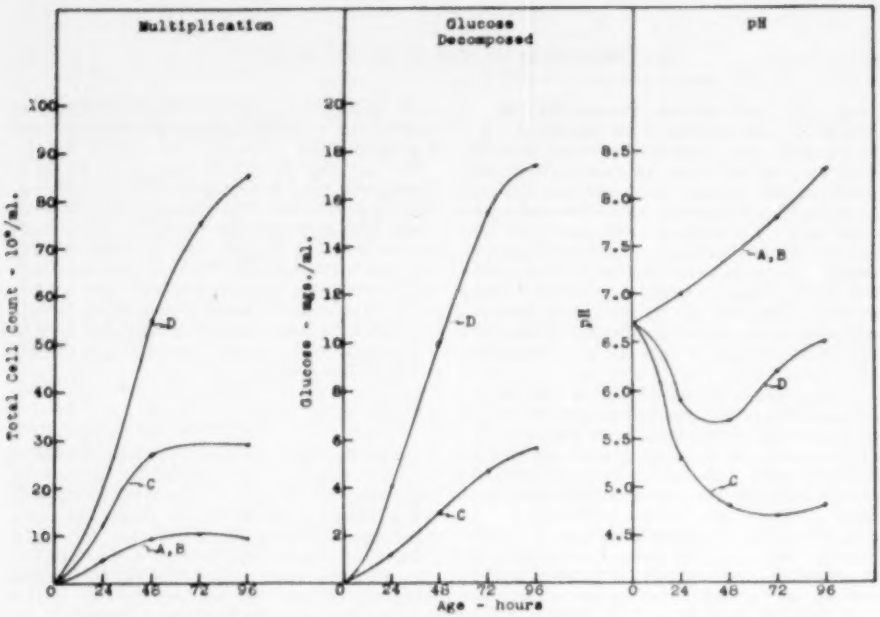

Fig. 5—Effects of thiamine and glucose on multiplication and metabolism of *Brucella melitensis*. Atmosphere: flowing oxygen. Medium: A = tryptose, 2 per cent; NaCl, 0.5 per cent. B = medium A + thiamine.HCl, 0.5 mg. per 100 ml. of medium. C = medium A + glucose, 2 per cent. D = medium B + glucose, 2 per cent.

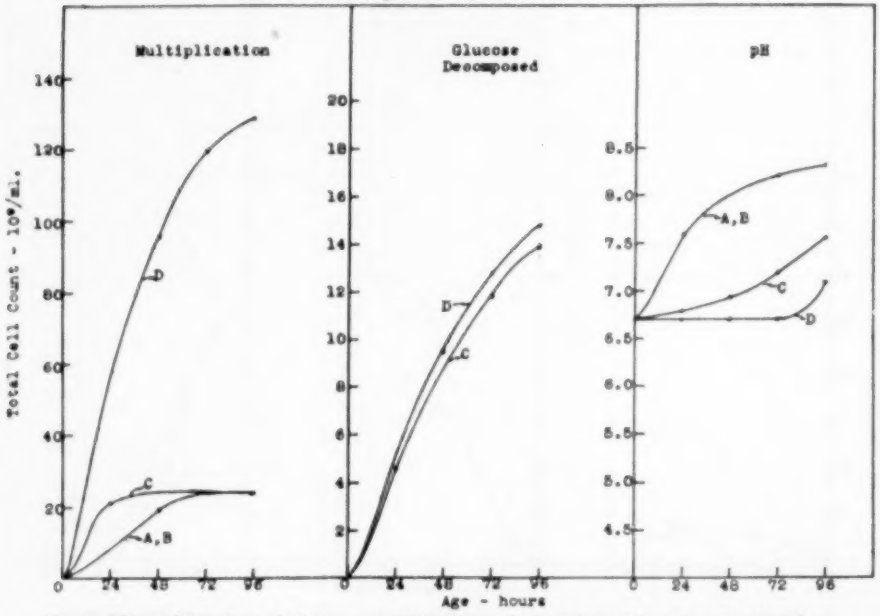

Fig. 6—Effects of thiamine and glucose on multiplication and metabolism of *Brucella suis*. Atmosphere: flowing oxygen. Medium: A = tryptose, 2 per cent; NaCl, 0.5 per cent. B = medium A + thiamine.HCl, 0.5 mg. per 100 ml. of medium. C = medium A + glucose, 2 per cent. D = medium B + glucose, 2 per cent.

by Brucella depended on the quality of the base medium.

The foregoing studies have demonstrated some of the factors which influence the growth and metabolic activities of Brucella organisms when the culture medium is agitated and exposed to pure oxygen during growth. One of the important effects produced by oxygen was an increase in the activity of the oxidative enzyme system that

TABLE 1—Decomposition of Glucose by Brucella*

	Tryptose** (%)	Glucose decomposed mg./10⁹ cells: Stagnant air	Glucose decomposed mg./10⁹ cells: Flowing oxygen
Brucella suis	1	0.12	0.24
	2	0.08	0.14
	3	0.06	0.10
Brucella abortus	1	0.35	0.28
	2	0.20	0.30
	3	0.16	0.25
Brucella melitensis	1	0.30	0.34
	2	0.18	0.25
	3	0.13	0.20

*Growth period: ninety-six hours.
**Medium: tryptose, glucose, 2 per cent; NaCl, 0.5 per cent; thiamine.HCl, 0.5 mg. per 100 ml. of medium.

was responsible for the decomposition of glucose by all three of the species.

In an atmosphere of pure oxygen, thiamine was not required for the initial attack on glucose, although it was needed for efficient utilization of the carbohydrate in producing large cell populations. *Brucella suis*, when grown in a medium without added thiamine, decomposed a large amount of glucose; when thiamine was added to the medium, the amount of glucose decomposed was not increased but the cell population was six times greater. In the case of *Br. abortus* and *Br. melitensis*, the addition of thiamine to the medium resulted in increases in both the amounts of glucose decomposed and the rate of multiplication.

The low minimums in the pH curves of *Br. abortus* and *Br. melitensis* suggested a transient accumulation of acids in cultures of these two species that was not shown by *Br. suis*. In order to account for the acidity in the medium and to examine for species differences in metabolism, studies were made of some of the possible intermediate products of glucose decomposition.

When pyruvic acid was substituted for glucose in the medium, it was decomposed but not at the same rate by each of the species. Although traces of pyruvic acid were detected in the medium containing glucose with no added thiamine, none was measured when thiamine was present. With all species, pyruvic acid appears to be a transitory product in the metabolism of glucose.

Lactic acid, substituted for glucose in the medium, was utilized by *Br. abortus* and *Br. suis* but not by *Br. melitensis*. The presence of lactic acid in cultures grown in a medium containing glucose was not demonstrated with any of the three species. If, in the process of metabolism, there was a dismutation of glucose or pyruvic acid to lactic acid, it would appear that further oxidation could proceed in cultures of *Br. suis* and *Br. abortus*.

Analyses demonstrated no acetic acid or other volatile acids in cultures of any of the species.

From early studies of metabolism of Brucella, McAlpine and Slanetz[11] reported that large amounts of free ammonia were produced by all strains in a liquid medium in the absence of glucose. On the other hand, in a medium containing glucose, strains of Brucella which utilized the carbohydrates produced only slight amounts of ammonia. They were unable to demonstrate the utilization of glucose by *Br. abortus*.

Collection and measurement of carbon dioxide showed that large amounts are evolved during metabolism of glucose by all three species of Brucella under the conditions described, and indicate that carbon dioxide is the major end product of glucose metabolism. The studies of possible intermediates of metabolism show that the species differ primarily in the rate of glucose decomposition.

A reciprocal sparing action of carbohydrate and peptone was shown by analyses

TABLE 2—Amounts of Ammonia and Carbon Dioxide Measured in Cultures of Brucella*

	Medium**	Ammonia (mg.)	Carbon dioxide (Gm.)
Brucella suis	0% glucose	181	1.4
	2% glucose	96	5.4
Brucella abortus	0% glucose	79	0.6
	2% glucose	42	5.0
Brucella melitensis	0% glucose	98	0.8
	2% glucose	66	4.8

*Growth period: seven days. Atmosphere: flowing oxygen.
**Medium: tryptose, 2 per cent; NaCl, 0.5 per cent; thiamine.HCl, 0.5 mg. per 100 ml. of medium; total volume, 250 ml.

of residual glucose and concurrent measurements of carbon dioxide and ammonia during growth of the three species in mediums with and without glucose. As the concentration of tryptose was increased in the medium, there was less glucose decomposed per unit number of cells, and, conversely, when carbohydrate was present, there was

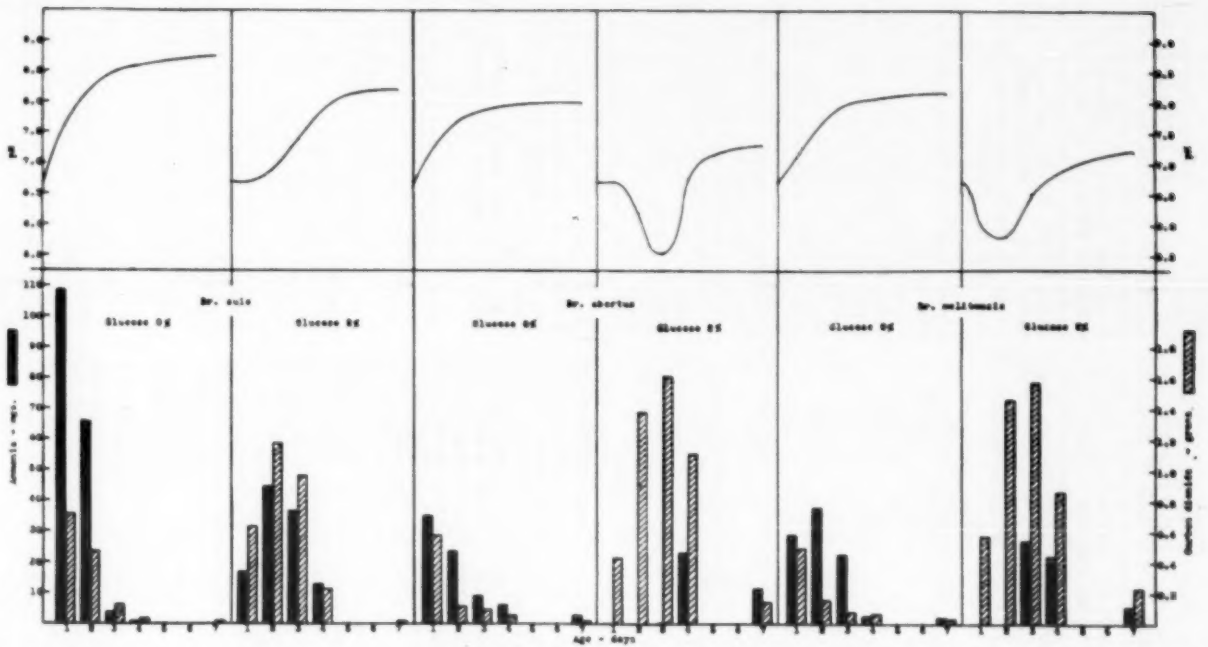

Fig. 7—Rates of production of ammonia and carbon dioxide and pH changes during growth of Brucella. Atmosphere: flowing oxygen. Medium: tryptose, 3 per cent; glucose; NaCl, 0.5 per cent; thiamine. HCl, 0.5 mg. per 100 ml. of medium.

less ammonia evolved. The preferential breakdown of carbohydrate over peptone decomposition was emphasized in cultures of *Br. abortus* and *Br. melitensis* by the production of an initial increase in acidity in the medium during the first two or three days of incubation when carbohydrate decomposition was rapid and no ammonia was formed. As the utilization of carbohydrate became slower, peptone decomposition was initiated and the pH of the culture medium increased as ammonia accumulated. The medium in which *Br. suis* was multiplying remained neutral-to-slightly-alkaline as a result of continued peptone breakdown simultaneous with carbohydrate decomposition. It is evident that the enzyme systems of *Br. suis* responsible for peptone metabolism perform more efficiently under the conditions employed than do those of *Br. abortus* and *Br. melitensis*. No basic variations in the metabolic processes of the three species were indicated; the chief differences were in the rates of the reactions.

SUMMARY

The metabolism of glucose by all species of Brucella, when grown as described, is a rapid oxidation of the carbohydrate. The major end product is carbon dioxide.

More glucose was decomposed per unit number of cells by all species when pure oxygen was supplied than when the medium was exposed to stagnant air. *Brucella suis* decomposed less glucose per unit number of cells than did the other two species.

Ammonia evolution was most rapid from cultures of *Br. suis* in all mediums. A sparing action of glucose for peptone was noted for all species but was much less pronounced for *Br. suis*. The pH changes that occur in mediums during the growth of Brucella emphasize the unreliability of any method of determining carbohydrate utilization that depends on detection of acidity.

The rates of reactions of the enzyme systems of Brucella account for the differences of the three species in the decomposition and metabolism of peptone, glucose, pyruvate, and lactate.

References

[1]Sanders, Evelyn, and Huddleson, I. F.: The Influence of Atmospheric Gases on the Multiplication of Brucella. Am. J. Vet. Res., *11*, (1950) 70-75.

[2]Benedict, S. R.: Determination of Blood Sugar. II. J. Biol. Chem., *76*, (1928):457-476.

[3]Clift, F. P., and Cook, R. P.: A Method of Determination of Some Biologically Important Aldehydes and Ketones, with Special Reference to Pyruvic Acid and Methylglyoxal. Biochem J., *26*, (1932):1788-1799.

[4]Elliot, K. A. C., Benoy, M. P., and Baker, Z.: The Metabolism of Lactic and Pyruvic Acids in Normal and Tumour Tissues. II. Rat Kidney and Transplantable Tumours. Biochem. J., *29*, (1935): 1937-1950.

[5]Friedemann, T. E., and Graeser, J. B.: The Determination of Lactic Acid. J. Biol. Chem., *100*, (1933):291-308.

[6]Friedemann, T. E.: The Identification and Quantitative Determination of Volatile Alcohols and Acids. J. Biol. Chem., *123*. (1938):161-184.

[7]Hill, G. M.: Aneurin and Pyruvate Metabolism by *Staphylococcus Aureus*. Biochem. J., *32*, (1938): 383-391.

[8]Quastel, J. H., and Webley, D. M.: Vitamin B_1 and Bacterial Oxidations. I. Dependence of Acetic Acid Oxidation on Vitamin B_1. Biochem. J., *35*, (1941):192-206.

[9]Kliger, I. J., Grossowicz, N., and Bergner, S.: Studies on the Role of Niacin and Thiamine in the Metabolism of Glucose by *Staphylococcus Aureus*. J. Bact., *46*, (1943):399-403.

[10]Grossowicz, N.: Growth Requirements and Metabolism of *Neisseria Intracellularis*. J. Bact., *50*, (1945):109-115.

[11]McAlpine, J. G., and Slanetz, C. A.: Studies on the Metabolism of the Abortus-Melitensis Group. 3. Glucose Utilization. J. Infect. Dis., *42*, (1928):73-78.

[12]ZoBell, C. E., and Meyer, K. F.: Metabolism Studies of the Brucella Group. VII. Dextrose Utilization. J. Infect. Dis., *51*, (1932):109-116.

[13]McNutt, S. H., and Purwin, Paul: Acidity Produced in Brucella Cultures. J. Infect. Dis., *48*, (1931):292-294.

Brucellosis in Male Guinea Pigs

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THE STUDY reported here was made in an attempt to ascertain: (1) the frequency and intensity of elimination of *Brucella suis* in the semen of male guinea pigs exposed by three different routes; (2) the transmissibility of brucellosis from infected male guinea pigs to noninfected female guinea pigs; and (3) the survival of *Br. suis* in guinea-pig semen.

REVIEW OF LITERATURE

There has been considerable difference of opinion during the past two decades in regard to the role of the male in the spread of brucellosis. It has been reported that *Brucella melitensis* may be transmitted by the male goat.[1] *Brucella abortus* organisms have been isolated from various regions of the genital tract of the bull.[2,3,4,5] Boars were found to be more frequently infected and more apt to transmit brucellosis than bulls.[6] *Brucella suis* organisms were recovered from the testes of 8 of 103 exposed animals[7] and evidence was presented of the actual spread of brucellosis by 1 infected boar. *Brucella suis* was recovered[8] from the genitalia of 11 of 19 boars which reacted to the agglutination test. *Brucella suis* was transmitted by 1 naturally and 2 experimentally infected boars. Weanling boar pigs can be readily infected when exposed by *Br. suis per os*, subcutaneously, *per rectum*, or intravenously.[9] *Brucella suis* was recovered[10,11] from 63 of 92 semen samples from 6 naturally infected boars and the semen quality of Brucella-infected boars appeared to be subnormal.

EXPERIMENTAL PROCEDURE

Three different methods were used to expose a total of 51 male guinea pigs to *Br. suis*. The animals were divided into five groups. The cultures of *Br. suis* used for exposure were grown for forty-eight hours on tryptose agar (Difco) slants and then suspended in physiologic saline solution. After exposure, the guinea pigs were kept in individual cages which had wire bottoms and solid sides.

Semen collections were made at weekly or biweekly intervals throughout the experiments. The semen was collected by means of the electrical stimulation method.[12] A needle electrode was pushed through the skin at the base of the skull, and a blunt electrode was placed in the guinea pig's mouth. The electrical stimulation did not last longer than ten seconds per stimulus. Two or three ejaculates were collected in succession, an ejaculate usually being obtained at each application of the electrical stimulus. The two or three ejaculates obtained in succession were considered a semen sample. One preliminary test collection was obtained and cultured two to three days prior to exposure of the guinea pigs to *Br. suis*.

The ejaculate was collected in sterile, shell vials. The samples were cultured by means of a sterile wire loop. Tryptose agar, adjusted to a pH of 6.7 to 6.9, was used as the culture medium in all cases. The inoculated plates were incubated from forty-eight to ninety-six hours at 37.5 C. All cultures recorded as positive gave a positive agglutination reaction with known Brucella serum and conformed to other tests for *Br. suis*. If, on culture, the semen proved to be contaminated but looked as though it might contain Brucella it was injected into guinea pigs.

After infection was established in the male guinea pigs, the concentration of *Br. suis* in the semen was determined by diluting 0.1 cc. of semen in physiologic saline solution, and 1-cc. amounts of different dilutions were placed on the surface of tryptose agar, Petri plates in triplicate. The number of colonies was counted after seventy-two hours' incubation.

Survival studies were made on semen samples held at 4 C. for varying periods; weekly bacteriologic examinations were made of the samples until no more *Br. suis* organisms were recovered.

Two strains of *Br. suis* were used: in groups 1 and 2, a strain obtained from Michigan State College; in groups 3 and 4, a strain recovered from a naturally occurring outbreak of swine brucellosis; and in group 5 a mixture of the two strains.

In group 1, 6 male guinea pigs (No. 1 to 6) were exposed to *Br. suis* by subcutaneous injection of 1 cc. of physiologic saline suspension containing approximately 7,000 organisms per cubic centimeter.

In group 2, 9 male guinea pigs (No. 7 to 15)

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were exposed by intraperitoneal injection of 1 cc. of a physiologic saline suspension containing approximately 7,000 organisms. Eleven weeks after exposure, the guinea pigs in groups 1 and 2 were sacrificed, blood samples were taken, and the following organs were examined bacteriologically for *Br. suis:* heart, lungs, liver, spleen, kidneys, bladder, seminal vesicles, testes, and epididymides. Agglutination tests were run on a sample of serum from each guinea pig, using standard plate antigen from the U. S. Department of Agriculture, Bureau of Animal Industry, in dilutions of 1 : 25, 1 : 50, 1 : 100, 1 : 200, and 1 : 400.

Four weeks after exposure of the males in groups 1 and 2, 2 females were placed with each male and left with them for approximately seven weeks or until the males were destroyed. The females were sacrificed for bacteriologic examination approximately four weeks after the males were removed. Liver, spleen, uterus, and cervical region of the vagina were examined bacteriologically for *Br. suis*. Blood samples were collected and serum agglutination tests were conducted as described previously for the males. Observations were made for pregnancy.

Twenty-one male guinea pigs were used in groups 3 and 4. Group 3 consisted of 11 males (No. 16 to 26) fed commercial pellets saturated with a heavy suspension of *Br. suis*. They had not been fed the day before and were fed individually on the day of exposure. Group 4 included 10 males (No. 27 to 36), each injected subcutaneously with 1 cc. of a physiologic saline suspension containing approximately 30,000 *Br. suis* organisms. These males were killed and examined bacteriologically for *Br. suis* nine weeks after exposure. Bacteriologic examination and serum agglutination tests were made as for groups 1 and 2.

In groups 3 and 4, 2 females were placed with each male, 1 at two weeks and 1 at four weeks after exposure of the males. The females were sacrificed and examined bacteriologically as in groups 1 and 2.

Fifteen males (No. 37 to 51) were used in group 5. They were all exposed by subcutaneous injection with 1 cc. of a physiologic saline suspension containing approximately 15,000 *Br. suis* organisms. The males were destroyed and examined for *Br. suis*, as in groups 1 and 2 five weeks after exposure. In addition to the organs cultured in the other groups, the prostate glands were cultured in group 5.

Six estrous females were placed with the infected males of group 5 for one day. Each female was then removed and kept in an individual cage until destroyed. Three other estrous females were artificially inseminated with semen naturally infected with *Br. suis*. Four females of undetermined estrual status were placed with 3 of the males and kept with them until the males were destroyed. All the females were destroyed three weeks after the males were brought to autopsy.

Tissues for histologic study were taken from all five groups of males.

RESULTS

Brucella suis was readily recovered by direct culture from the semen of guinea pigs exposed to the organisms by the three routes, *i.e.*, subcutaneous, intraperitoneal, and oral.

In group 1, exposed subcutaneously, *Br. suis* was recovered from the semen of 3 of the 6 guinea pigs. Two males died prior

TABLE 1—Summary of the Results of Exposure of Male Guinea Pigs to *Brucella Suis*

Group No.	1	2	3	4	5
Method of exposure	s.c.*	i.p.**	*per os* saturated feed	s.c.	s.c.
Exposure dose	7,000	7,000		30,000	15,000
No. of guinea pig males	6	9	11	10	15
No. eliminating *Br. suis* in semen	3	7	4	9	7
No. semen samples collected	44	76	75	68	49
No. semen samples containing *Br. suis*	14	33	13	18	15
Earliest semen recovery of *Br. suis*	29 days	14 days	30 days	20 days	21 days
No. examined bacteriologically at postmortem	2	5	10	7	8
No. positive for *Br. suis* at postmortem	2	5	4	7	8
Recovery of *Br. suis* from urogenital tract					
Kidney	2	2	2	3	5
Bladder	1	2	4	5	7
Seminal vesicles	1	2	4	4	6
Testes	1	5	3	4	7
Epididymides	1	4	3	6	6
Prostate	..	..	..	..	7
No. females placed with males	8	16	20	18	10
No. females infected	0	0	0	0	9
No. females artificially inseminated					3
No. females infected					1

*s.c. = subcutaneous injection. **i.p. = intraperitoneal injection.

to the recovery of *Br. suis* from the semen and 1 failed to eliminate the organisms. Elimination of the organisms in the semen began on the twenty-ninth day after exposure in 2 and on the thirty-fifth day in 1 guinea pig. The organisms were recovered from the semen repeatedly until the males were destroyed seventy-seven days after exposure and from 14 of a total of 44 semen samples in group 1.

Postmortem examination of 2 males revealed the usual lesions of brucellosis. *Brucella suis* was isolated from both of these guinea pigs, and the agglutination test was positive in both cases. The remaining 4 either died or were not examined postmortem.

None of the females placed with the males in group 1 became pregnant, developed an agglutination titer, or showed any lesions; nor was *Br. suis* recovered from any of the tissues examined bacteriologically.

In group 2, exposed intraperitoneally, 7 of the 9 guinea pigs eliminated *Br. suis* in the semen. Two died prior to the recovery of the organism from the semen. Elimination of *Br. suis* in the semen began on the fourteenth day after exposure in 1 male and not until the fiftieth day after exposure in 1 male. The organisms were recovered repeatedly from the semen of all 7 guinea pigs until they were either removed from the study or were destroyed for bacteriologic examination seventy-seven days after exposure. *Brucella suis* was recovered from 33 of 76 semen samples in group 2.

Postmortem bacteriologic examinations of 5 males in group 2 were positive, as were the agglutination tests of their serums. Two males died during the course of the study and were not cultured. Two were not brought to autopsy.

None of the females placed with the males of group 2 became infected as evidenced by negative bacteriologic findings for *Br. suis* and negative serum agglutination responses at autopsy.

In group 3, exposed to *Br. suis* by means of the feed, only 4 of the 11 guinea pigs ejaculated infected semen. Elimination of *Br. suis* in the semen was recorded on the thirtieth day after exposure in these 4 guinea pigs and continued until the study was terminated seventy-three days after exposure, as well as in 13 of the 75 semen samples collected.

On postmortem examination of 10 of the 11 guinea pigs in group 3, *Br. suis* was recovered from the tissues of 4. A positive agglutination titer was demonstrated in only 1. One guinea pig died and was so decomposed that no bacteriologic examination was made. Five males in this group yielded neither *Br. suis* from their tissues nor positive agglutination responses from their blood.

None of the females placed with the males of group 3 developed a serum agglutination titer, nor was *Br. suis* recovered from any of the tissues examined at autopsy. Three females placed with 3 males, after exposure of the males, became pregnant, but *Br. suis* was not recovered from any of these three males.

In group 4, exposed subcutaneously, *Br. suis* was recovered from the semen of 9 of the 10 males. Elimination of the organisms in the semen was observed in 4 males on the thirtieth day; in 2 on the thirty-eighth day; 2 on the fifty-first day; and 1 on the sixty-second day after exposure; and *Br. suis* was isolated from 18 of 68 semen samples.

Three males in group 4 died during the study and were not examined bacteriologically because of decomposition. All of the remaining 7 guinea pigs in this group yielded *Br. suis* from various tissues and showed high-titered serum agglutination reactions at autopsy.

Of the females placed with the males of group 4, only 1 was pregnant. This female was placed with the male two weeks after his exposure. Elimination of *Br. suis* in the semen of this male occurred fifty-one days after exposure. The pregnancy was well advanced. All bacteriologic examinations for *Br. suis* on this female and the others in the group were negative.

In group 5, exposed subcutaneously, *Br. suis* was isolated from the semen of 7 of the 15 guinea pigs. Elimination of the organisms was first observed on the twenty-first day after exposure from 3, the thirtieth day from 3, and the fortieth day from 1, while *Br. suis* was recovered from 15 of 49 semen samples.

Eleven males died before the termination of the study, and *Br. suis* was recovered from 4 of these 11. The others were too decomposed for bacteriologic examination. Four males were destroyed fifty-three days after exposure and *Br. suis* was recovered from all 4.

Two estrous females were placed with 1 male and 1 estrous female with each of 4 males in group 5. Mating was not observed in any of the females nor was *Br. suis* recovered from these females. One female of unknown estrual status was placed with each of 2 males, and 2 females of unknown estrual status were placed with 1 male of group 5. *Brucella suis* was not recovered from these 4 females. Three

females were artificially inseminated with Brucella-infected semen from 3 different males. The organism was not recovered from 2 females. The other female, inseminated with naturally infected semen, became infected as evidenced by positive serum agglutination and the recovery of *Br. suis* from the spleen, kidney, inguinal lymph glands, uterus, and cervix.

The gross appearance of semen from infected guinea pigs was considerably different than that of normal semen. Normal semen is milky in color and quite fluid; the semen from infected animals is frequently yellowish in color, curdled in appearance, or watery with white, flocculent particles. The secretion from the seminal vesicles of a normal guinea pig is rather translucent and white, and becomes firm a few minutes after ejaculation, while that of an infected guinea pig may fail to become firm, loses its translucency, and becomes yellowish in color.

Microscopically, the semen from infected guinea pigs showed considerable variation regarding the condition of the spermatozoa. Some samples showed many active spermatozoa, others contained few, and in some cases no motile spermatozoa. In the latter case, a great number of leucocytes, predominately polymorphonuclear, were present in the semen.

The concentration of *Br. suis* was determined for 19 samples of semen. The plate counts ranged from approximately 3,300 to 6,120,000 organisms per 0.1 cc. Five of the 19 had approximately 1 million or more organisms per 0.1 cc.

In two semen samples, *Br. suis* remained alive for forty-nine days when held at 4 C. This was the longest survival time recorded from any of the samples tested under these conditions.

The earliest observed elimination of *Br. suis* in the semen in these experiments began fourteen days after exposure in 1 male. One male eliminated *Br. suis* in eight consecutive semen samples beginning twenty-one days after exposure and continuing until the animal was killed, seventy-seven days after exposure.

Of the 51 males exposed, 36 were examined bacteriologically and *Br. suis* was not recovered from 10 of these. Of the remaining, 4 disappeared and 11 died, probably as a result of repeated electrical stimulation, and were too decomposed to culture. Of the 26 animals from which *Br. suis* was recovered, 15 recoveries were made from the spleen, 12 from the liver, 14 from the kidneys, 19 from the urinary bladder, 18 from the seminal vesicles, 20 from the epididymides, and 20 from the testes. The prostates of 8 infected males were cultured, and seven were found to be infected with *Br. suis*. It was observed also that the growth of the organisms seemed greater on many of the plates seeded from the testes, bladder, and prostate than on plates seeded from the other organs.

That the genital organs were heavily infected was also shown by the presence of as many as 6 million organisms in 0.1 cc. of semen.

The multiplication of *Br. suis* in the male reproductive tract is indicated by the increased number of organisms recovered from the semen at different times. In 1 male, 3,300 organisms were recovered from 0.1 cc. of semen collected fifty-six days after exposure; fourteen days later, approximately 1,326,000 organisms per 0.1 cc. of semen were recovered from the same animal. In 2 other males, the results were not so striking. In 1, fifty-six days after exposure, a semen sample contained approximately 1,650,000 organisms per 0.1 cc.; fourteen days later, 6,120,000 organisms per 0.1 cc. of semen were recovered from the same animal. Another male eliminated approximately 32,400 organisms per 0.1 cc. of semen thirty days after exposure, and, twenty-one days later, a semen sample contained 50,000 organisms per 0.1 cc.

Histologic sections of the tissues of infected males in groups 1, 2, 3, and 4 showed granulomatous areas in the livers and lymph nodes. Necrotic areas and abscesses were prominent in the lymph nodes, liver, and spleen. There was round cell infiltration in these organs and in the urogenital organs. The lungs of 2 animals showed marked cellular infiltration around the bronchioles and blood vessels. Spermatogenesis had ceased in many of the testes and the interstitial cells were practically absent. There were many abscesses in the testes and epididymal regions. The seminal vesicles showed some round cell infiltration, but it was not marked. The heart, adrenal, stomach, and intestine did not show any microscopic lesions. Histologic sections of the testes, epididymides, and prostates from group 5 showed marked purulent infiltration and edema. There was metaplasia of epithelium in the epididymal region. A summary of these findings is given in table 1.

DISCUSSION

From the results, it appears that *Br. suis* readily localizes in one part or all parts of the reproductive system of infected male guinea pigs. Elimination of the organisms in the semen may follow localization in the

testes, epididymides, seminal vesicles, or prostate.

When the reproductive tract became infected, the males seemed to lose sex desire and the semen quality decreased; some males apparently produced no live spermatozoa. Loss of sex desire might explain the lack of pregnancy and failure of transmission of the infection to the females placed with the males. Only 1 female placed with infected males became pregnant. This was the female placed with a male two weeks after exposure of the male, and this mating probably occurred soon after the female was placed with the male because the pregnancy was almost to term at the time the female was destroyed. Organisms were not recovered from the semen of this male until fifty-one days after exposure.

The general condition of many of the males was poor. They lost much weight; the hair was sparse. This poor condition does not ordinarily occur in guinea pigs infected with Brucella. The combination of the electrical stimulation and the infection may have brought about the observed poor general condition. The electrical stimulation also may have caused lessening of sex desire and the numerous deaths which occurred.

It may be surmised that, under the conditions in which the guinea pigs were kept, pen transmission of *Br. suis* does not readily occur. Considering the heavy infection in the bladder in some of the males, it is probable that they were eliminating large numbers of *Br. suis* organisms in the urine. None bf the females kept with the males became infected with *Br. suis*.

The only female to which the infection was transmitted was 1 artificially inseminated with infected semen. A generalized infection with a complete agglutination at 1 : 400 dilution resulted.

Apparently, guinea-pig semen at refrigerator temperature has little or no bactericidal activity against *Br. suis*. The organisms remained alive as long as forty-nine days in naturally infected semen stored in the refrigerator.

There was little difference in the results from intraperitoneal and subcutaneous routes of exposure. The feeding method was not as effective as the other two in establishing an infection.

SUMMARY

1) *Brucella suis* was recovered from the semen of 30 of 51 guinea pigs exposed to the organisms experimentally by the following routes: subcutaneous injection, intraperitoneal injection, and feeding.

2) The concentration of *Br. suis* in the semen of infected males was high. One male eliminated approximately 6,000,000 organisms in 0.1 cc. of semen.

3) Guinea-pig semen had little or no bactericidal action against *Br. suis* at refrigerator temperature. Organisms remained viable for as long as forty-nine days in naturally infected semen stored at 4 C.

4) Infected males failed to transmit the infection to females placed with them for several weeks. However, 1 female became infected when artificially inseminated with naturally infected semen. A generalized infection resulted in this female.

5) Histologic sections of tissues showed abscesses, round cell infiltration in lymph nodes, livers, spleens, testes, and epididymides. Spermatogenesis had ceased in many of the seminiferous tubules, and interstitial cells were scant.

References

[1]Dubois, Ch.: La fièvre de Malte chez les animaux domestiques. Revue vétérinaire, *36*, Annee, (1911): 129.

[2]Barendregt, A. A.: Een minder bekende vorm van abortusverspreiding bij het rund en haar eventueele bestrijding. Tijdschr. voor Beeartsenijkunde, *40*, (1913): 691.

[3]Buck, J. M., Creech, G. T., and Ladson, H. H.: *Bacterium Abortus* Infection of Bulls. J. Agric. Res., *17*, (1919): 229.

[4]Bendixen, H. C.: A Case of Infection with *Brucella Abortus* in Ampulla Ductus Deferentis in a Bull. Maanedsskr. Dyrl., *56*, (1944a): 1-11.

[5]Bendixen, H. C., and Blom, Erik: Investigations on Brucellosis in the Bovine Male with Special Regard to Spread of the Disease by Artificial Insemination. The Vet. J., *103*, (1947): 337-345.

[6]Cotton, W. E., and Buck, J. M.: Bureau of Animal Industry Researches on Infectious Abortion. J.A.V.M.A., *31*, (1931): 306.

[7]Graham, R., Boughton, I. B., and Tunnicliff, E. A.: Studies on Porcine Infectious Abortion. Univ. of Illinois Agric. Exper. Sta., Bull. 343, (1930): 298.

[8]Thompson, Axel.: Brucella Infection in Swine. Acta. path. et microbiol. Scandinav., Supplementum 21, 1934. (Translation by Hans Anderson, M.D.)

[9]Hutchings, L. M., Delez, A. L., and Donham, C. R.: Studies on Brucellosis of Swine. I. Infection Experiments with Weanling Pigs. Am. J. Vet. Res., *5*, (1944): 195.

[10]Hutchings, L. M., and Andrews, F. N.: Studies on Brucellosis in Swine. III. Brucella Infection in the Boar. Am. J. Vet. Res., *7*, (1946): 379-384.

[11]Andrews, F. N., and Hutchings, L. M.: Studies on Brucellosis in Swine. IV. Semen Quality in Brucella-Infected Boars. Am. J. Vet. Res., *7*, (1946): 385-387.

[12]Battelli, F. Une methode pour obtenir l'emission complete du liquide des vesicules seminales chez le cobaye. Compt. rend. Soc. de physique et d'hist. nat. de Genève, *39*, (1922): 73-74.

Effect of Aureomycin on *Leptospira Canicola* and *Leptospira Icterohaemorrhagiae in Vitro* and Experimental Carrier Studies

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STREPTOMYCIN was used[1] in the treatment of Leptospira carriers and the results indicated that this antibiotic effectively eliminated *Leptospira canicola* from the kidneys of experimentally infected hamsters and dogs. Again, the satisfactory method of creating experimental *L. canicola* carriers by treating young hamsters with penicillin shortly after infection was used. Instead of streptomycin, which had proved to be equally effective with hamsters and dogs, aureomycin was tested. The experiment with *L. canicola* reported herein gave such uniformly good results that the efficiency of aureomycin against this organism seems established. Treatment of experimental *Leptospira icterohaemorrhagiae* infection with this drug was also successful.[2] Although its therapeutic value is probably of the same order as that of streptomycin in these infections, it offers the advantage of oral administration.

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METHODS

In Vitro Studies with Leptospira Canicola and Leptospira Icterohaemorrhagiae.—A preliminary *in vitro* test proved the strongly inhibitory effect of aureomycin on *L. canicola* and *L. icterohaemorrhagiae*. Decreasing concentrations of the antibiotic were tested. For each concentration, 2 tubes containing 2 cc. of Schüffner's medium (Verwoort's modification) were inoculated with 20,000,000 organisms per cubic centimeter of medium and incubated at 30 C.

Virulent seven-day cultures of *L. canicola* strain 1126 (passed on young hamsters) and virulent thirteen-day cultures of *L. icterohaemorrhagiae* strain 917 (passed on young guinea pigs) were used as inoculums.

Concentrations of 0.1 μg. per cubic centimeter of aureomycin gave complete inhibition; 1.0 μg. per cubic centimeter was bactericidal after fifty to sixty-four hours' incubation (table 1). If the organisms lost motility and subcultures were negative, the drug was considered bactericidal.

TABLE 1—Effect of Aureomycin *in Vitro*

Leptospira type	Aureomycin (μg./cc.)	Movement* and growth** after Hours 18	30	42	50	64	90	Days 10	30
Canicola	1,000	—	—	—	—	—	—	—	—
Ictero-haemorrhagiae		—	—	—	—	—	—	—	—
Canicola	100	+	+	+	—	—	—	—	—
Ictero-haemorrhagiae		+	—	—	—	—	—	—	—
Canicola	10	+ + + +	+ + +	+ +	+	—	—	—	—
Ictero-haemorrhagiae		+ + + +	+ + +	+	+	—	—	—	—
Canicola	1	+ + + +	+ + +	+ +	+ +	—	—	—	—
Ictero-haemorrhagiae		+ + + +	+ + +	+ +	+	—	—	—	—
Canicola	0.1	+ + + +	+ + + +	+ + +	+ + +	+ + +	+ + +	+ + +	+
Ictero-haemorrhagiae		+ + + +	+ + + +	+ + +	+ + +	+ + +	+ + +	+ + +	+
Canicola	0	+ + + +	g	g	g	dg	dg	dg	dg
Ictero-haemorrhagiae		+ + + +	+ + + +	g	g	g	g	g	g

*+ + + + = all moving; + + + = some dead; + + = some moving; + = few moving. **g = growth; dg = dense growth.

In Vivo Studies on Leptospira Canicola Carriers (Hamsters).—Experiments with hamsters were begun by inoculating 33 hamsters, 5 weeks old, intraperitoneally, with 0.25 cc. of a virulent *L. canicola* culture. Penicillin treatment after sixty and seventy-two hours (500 units intramuscularly in oil and wax) proved to be too late to save the animals; 1 hamster died even before the second penicillin injection; 15 died after six days; and 13 died between the sixth and the twelfth days. The sixty-hour interval between infection and treatment had been chosen on the basis of a previous experiment. Thereafter, constant passing of this strain had decreased the interval between infection and death from five to four days; hence earlier treatment was necessary for this work.

Fifty hamsters of the same age were then inoculated intraperitoneally with 0.1 cc. of a virulent culture of *L. canicola* (about 120,000 organisms/cc.) and were treated with penicillin (500 units in oil and wax) after forty-three and fifty-seven hours. Five hamsters died of leptospirosis from the eighth to the twelfth days after infection.

Of the remaining 45 hamsters, 10 were killed after 30 days; kidney material was examined in the dark field and cultured. All cultures were positive, and in every case the organisms could readily be demonstrated in the fresh material.

Aureomycin* treatment of 25 hamsters was begun the same day; 1 mg. in saline was given intraperitoneally every eight hours for three days to 15 hamsters and for five days to 10 hamsters. Ten were left untreated as further controls. Forty-eight hours after the last treatment, all the 35 remaining hamsters were killed and kidney material was examined and cultured. Results of examination of the 10 control hamsters were again all positive, while the cultures of the 25 treated animals remained sterile.

Because the experimental work with hamsters reflected the value of aureomycin, it was used to treat a few dogs. Practical considerations made treatment of a large series impossible. Four young dogs, each weighing 3 or 4 kg., were experimentally infected with *L. icterohaemorrhagiae*. Seventeen days later, all 4 were shedding living leptospiras in the urine. At that time, 2 dogs were given 20 mg. of aureomycin orally every twelve hours for three days; the other 2 were not treated at this time, but were observed. Two days after the last treatment, the urine of all 4 dogs was examined, and while the 2 untreated dogs were still shedding the organisms, the urine of only 1 of the treated dogs still contained a few living leptospiras. The same result was found three days later. The 2 infected dogs that had not received the drug were then each treated with 150 mg. orally every twelve hours for three days. On the fifth day, leptospiras could not be found in the urine of these dogs. One month after treatment with the smaller dose was begun, urine from all 4 dogs was examined; the same dog that was receiving the smaller dose of aureomycin, now being considered inadequate, was still shedding leptospiras. This small series speaks for the use of adequately large doses to prevent development of the carrier stage.

*The antibiotic was supplied through the courtesy of Dr. Herald A. Cox, Lederle Laboratories, Pearl River, N. Y.

SUMMARY AND CONCLUSIONS

The influence of aureomycin on *Leptospira canicola* and *Leptospira icterohaemorrhagiae* was tested *in vitro*. One-tenth microgram per cubic centimeter of medium inhibited growth of both types completely, while 1.0 μg. per cubic centimeter was bactericidal after fifty to sixty-four hours.

The therapeutic effect of aureomycin on experimental Leptospira carriers (young hamsters and dogs) was tested. Of 50 young hamsters, 45 survived infection with *L. canicola* after treatment with penicillin. Of these, 20 chosen at random and killed after thirty and thirty-seven days proved to be renal carriers; while at the same time, 15 treated for three days and 10 treated for five days with aureomycin (1 mg. every eight hours, intraperitoneally in saline) proved to have sterile kidneys two days after the last treatment. Treatment of 4 young dogs shedding leptospiras, 2 with about 5 mg. and 2 with 40 mg. per kilogram body weight given orally, proved the therapeutic value of aureomycin in the larger dose.

References

(1) Brunner, K. T., and Meyer, K. F.: Streptomycin in the Treatment of Leptospira Carriers. Experiments with Hamsters and Dogs, Proc. Soc. Exptl. Biol. and Med., *70*, (1949): 450-452.

(2) Heilman, F. R.: Aureomycin in the Treatment of Experimental Relapsing Fever and Leptospirosis Icterohaemorrhagica (Weil's Disease), Proc. Staff Meeting, Mayo Clin., *23*, (1948): 569-573.

Studies on Blood Plasma of Domestic Animals
I. Absorption Spectrums of Plasma of Some Individual Animals

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THIS IS the introductory report on an extended investigation of pathologic and physiologic variations of animal plasma by measurement of its light absorption characteristics. The general purposes of the investigation are: (1) to determine the light-absorption characteristics of normal plasma of the principal domestic animals; (2) to study variations in the absorption characteristics of normal plasma due to physiologic variations; (3) to study variations of absorption characteristics of plasma under pathologic conditions with a view of applying these data to clinical diagnostic procedures, and (4) to study variations of absorption characteristics of plasma as a result of the use of various therapeutic agents.

This report is concerned with the absorption characteristics in the spectral region 305-to 625-mμ wave length of plasma from various, clinically healthy, domestic animals. (The visible spectral region is approximately 400-to 700-mμ wave length.) It is not intended that the curves published in this report represent the standard absorption curves for the species. The absorption curves apply only to the individual animals used. The curves are presented to show: (1) the variations that might occur in an individual animal over a limited period of time, and (2) a comparison between absorption curves of plasma from individual animals of different species.

It is common observation that plasma of different domestic animals range from intense yellow in the cow and horse to nearly colorless in the cat and dog. It has been long recognized[1] that blood affords a sensitive index to the metabolic processes within the animal body. Gross changes in the physical or chemical characteristics of the blood brought about by pathologic conditions have been extensively used in clinical diagnostic procedures.[2,3] The clinical application of this principle is limited by insufficient knowledge of both physiologic and pathologic composition of the blood and methods of detecting component variations. The components of blood primarily responsible for the color of plasma[3,4] are bilirubin, hemoglobin, and carotene. The difference in color of the plasma of the various animals is due to variations in concentration of these three components.

The study of the absorption characteristics of the various components of blood is not new. Beginning with the classic work of Hoppe-Seyler[5] in 1868, numerous investigators have applied this physical measurement to the study of human blood. The absorption spectrums for hemoglobin and closely related derivatives have been thoroughly investigated by Drabkin and Austin,[6] Davis and Sheard,[6] and many others.[7,8,9,10,11] Bilirubin, a decomposition product of hemoglobin, has been studied from the aspect of absorption spectrums almost as thoroughly as that of hemoglobin.[12,13,14,15,16] The blood proteins do not contribute to the visual color of blood plasma; however, these proteins do possess distinct absorption characteristics in the ultraviolet (220- to 300-mμ wave length) as shown by Smith *et al.*[17,18,19] By far, the greater number of the above investigations have been made on human blood; however, a few investigators have utilized blood from domestic animals and shown a comparison between human and animal blood.[9,17] It is well to note that very little information has been published on spectrums of blood from the principal domestic animals from a comparative standpoint or with the view of its application to veterinary medicine.

MATERIALS AND METHODS

The animals selected for this investigation were clinically healthy. A brief description of each animal and the technique of sample collection follow.

Cow.—Jersey cow, 6 years old, nonpregnant, and lactating. This cow calved one month prior to being used for this experiment. Samples were obtained from the jugular vein.

Horse.—Clydesdale gelding, 11 years old, weighing 1,800 lb. Samples were obtained from the jugular vein.

Chicken.—White Leghorn, 5 to 7 weeks old (samples 1, 2, 3). Rhode Island Red, 8 weeks old (sample 4). The chickens were bled from the heart. Samples 1, 2, and 3 were from individual chickens from the same flock.

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Sheep.—Mature Rambouillet ewe. Samples were obtained from the jugular vein.

Dog.—Male Springer Spaniel, 5 years old (samples 1, 2, 3). Four-year-old Collie-type female (sample 4). Samples were obtained from the jugular vein.

Cat.—Mature male cat. Samples were obtained by bleeding directly from the heart.

Pig.—Chester White gilt, 8 months old. Samples were obtained from the anterior vena cava according to the method of Carle and Dewhirst.[29]

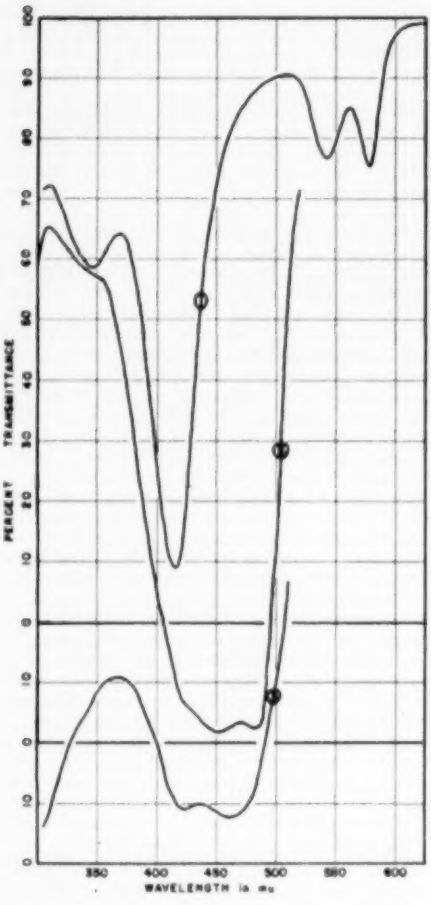

Fig. 1—Spectral transmittance curves for the color components of blood plasma.

1) Hemoglobin; whole blood from horse diluted 1:1,000 with 0.1 per cent Na_2CO_3. Concentration of hemoglobin 12 mg. per 100 cc. of blood plasma.

2) β - carotene; in hexane. Data recalculated from curves published by L. Zechmeister.[28] Concentration estimated to be 60γ per 100 cc. of blood plasma.

3) Bilirubin; in dog serum. Data recalculated from a curve published by Cutten.[27] Concentration estimated to be 80γ per 100 cc. of blood plasma.

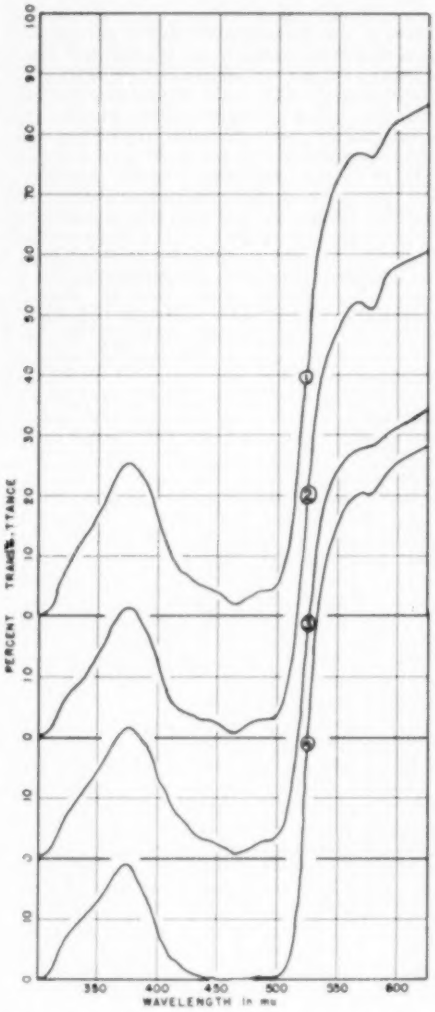

Fig. 2—Spectral transmittance curves for cow plasma.

Curve	Sampling date	Curve	Sampling date
1	Feb. 1, 1949	3	Feb. 15, 1949
2	Feb. 8, 1949	4	Feb. 22, 1949

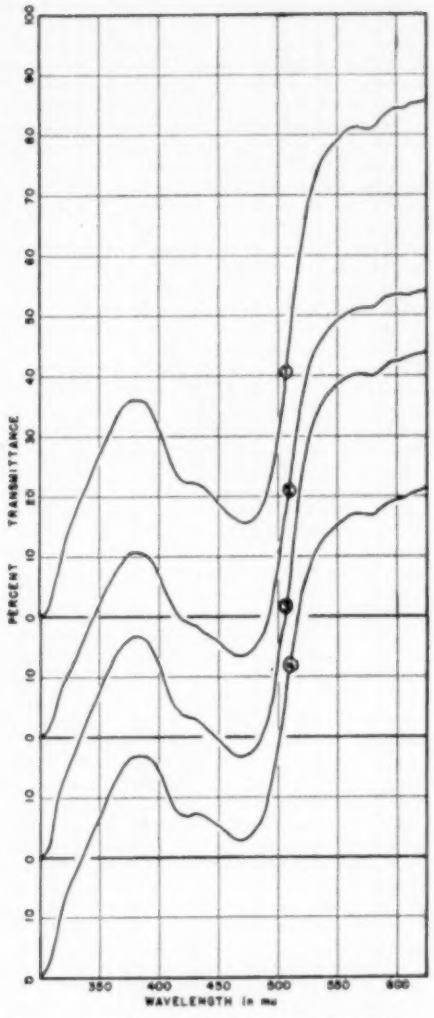

Fig. 3—Spectral transmittance curves for horse plasma.

Curve	Sampling date	Curve	Sampling date
1	Feb. 1, 1949	3	Feb. 15, 1949
2	Feb. 8, 1949	4	Feb. 22, 1949

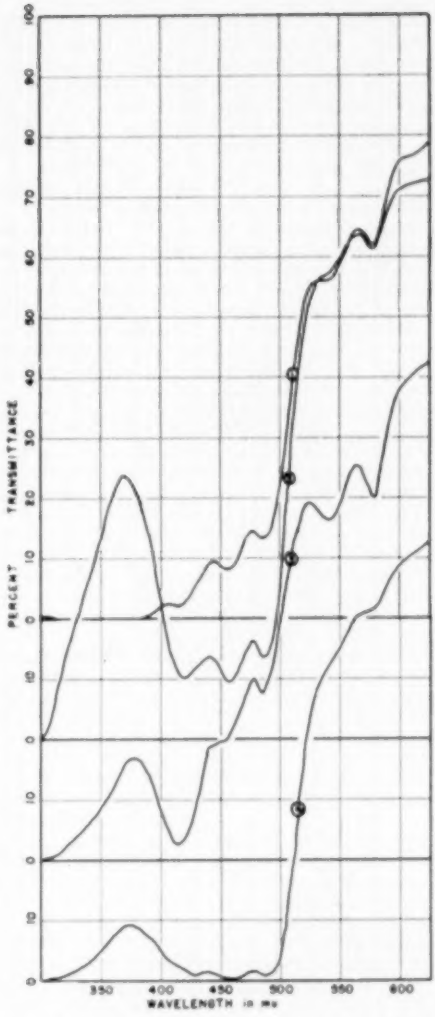

Fig. 4—Spectral transmittance curves for chicken plasma.

Curve	Sampling date	Curve	Sampling date
1	Feb. 3, 1949	3	Feb. 17, 1949
2	Feb. 10, 1949	4	Feb. 24, 1949

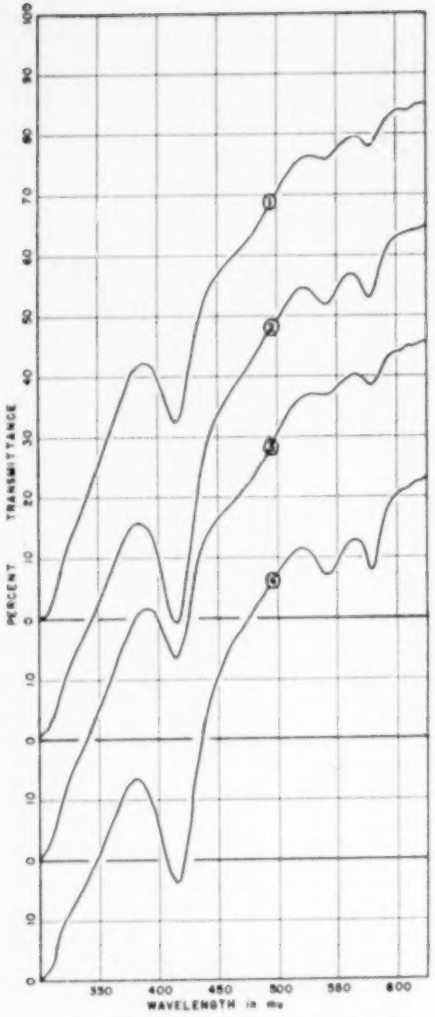

Fig. 5—Spectral transmittance curves for sheep plasma.

Curve	Sampling date	Curve	Sampling date
1	Feb. 1, 1949	3	Feb. 15, 1949
2	Feb. 8, 1949	4	Feb. 22, 1949

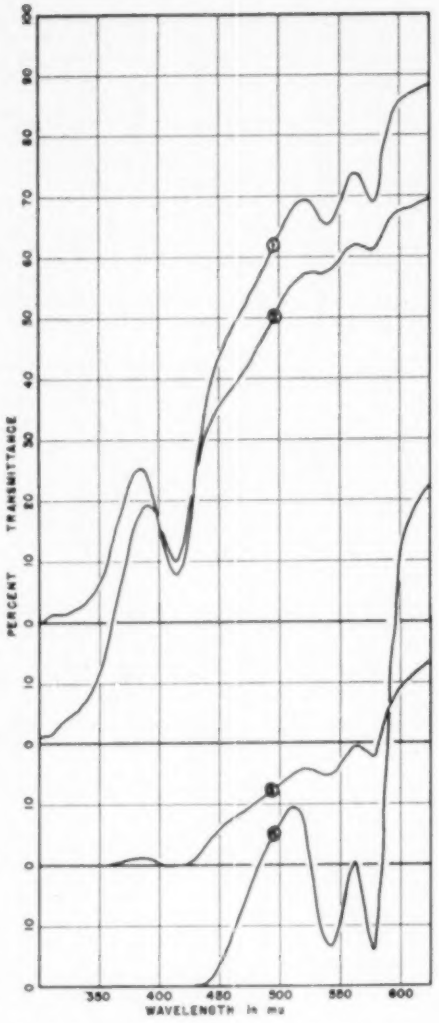

Fig. 6—Spectral transmittance curves for dog plasma.

Curve	Sampling date	Curve	Sampling date
1	Feb. 1, 1949	3	Feb. 15, 1949
2	Feb. 8, 1949	4	Feb. 22, 1949

The animals were sampled approximately two hours after the morning feedings, at weekly intervals over a period of four weeks. All samples were collected, using aseptic technique, by drawing the blood into a 20-cc. glass syringe containing two to three drops of heparin*. This amount of heparin was sufficient to moisten the barrel of the syringe and to prevent clotting of the blood. The plasma was obtained by centrifugation of the heparinized blood immediately after collection. The method of plasma preparation is a modification of the procedure outlined by Knacke.[8]

The spectral transmittance curve of the plasma was determined within a few hours after its preparation. A Beckman model DU quartz spectrophotometer was used to measure the absorption characteristics. The samples of plasma were analyzed in quartz cuvettes 1.000± .002 cm. in thickness. The blank used for comparison was distilled water unless otherwise indicated. The spectral transmittance readings were taken at 5-mμ intervals from 305 to 625 mμ. Preliminary measurements showed that the undiluted plasma samples gave complete light absorption in the ultraviolet region from 220 to 330 mμ. In the region from 625 to 2,000 mμ, the spectral transmittance was relatively constant at or near 90 per cent and thus would have very little significance in the analysis of the absorption characteristics. The spectral transmittance curve of a heparin solution of a concentration approximating that in the plasma samples indicated no absorption in the significant regions of plasma absorption. The absorption data were plotted as spectral transmittance (per cent transmittance versus wave length).

DISCUSSION

The absorption curves for hemoglobin, carotene, and bilirubin are illustrated in curves 1, 2, and 3, respectively (fig. 1). The hemoglobin was prepared by diluting whole blood 1 : 1,000 with 0.1 per cent Na_2CO_3, using a 1 : 1,500 dilution of plasma from the same whole blood as reference blank. This reference blank was used so that the absorption curve would represent the absorption due to hemoglobin alone. The hemoglobin curve shows the β and α absorption bands at 542 mμ and 578 mμ, respectively, a strong absorption maximum at 415 mμ, and a weaker absorption maximum at 345 mμ. Curve 2 shows the absorption characteristics of β-carotene in hexane with absorption maximums at approximately 485 mμ and 450 mμ. Data for this curve were recalculated from a curve published in an article by L. Zechmeister.[9] Curve 3 is the absorption curve for bilirubin taken from data published by Cutten.[10] The curve was obtained from a solution prepared by dissolving 1.6 mg. of bilirubin in 100 cc. of dog serum. Examination of the curve indicates absorption maximums at approximately 420 mμ and 465 mμ.

The absorption curves for the various animals are shown in figures 2 to 8, inclusive. Cow plasma (fig. 2) shows absorption maximums at 420, 465, 490, and 578 mμ. By reference to figure 1, it can be seen that the plasma contains a very small amount of hemoglobin (420 and 578 mμ), bilirubin (420 and 465 mμ), and a small amount of carotene (490 mμ). Visual observation of the blood plasma did not indicate any hemolysis.

The horse plasma (fig. 3) has a visual color very similar to that for the cow; however, examination of the absorption spectrums shows absorption maximums at 420, 465, and 578 mμ, but none at 490 mμ. Again, reference to figure 1 would indicate a very small amount of hemoglobin, a relatively large amount of bilirubin, and little, if any, carotene. As in the cow, no hemolysis was observed visually.

The absorption characteristics of chicken plasma (fig. 4) show absorption maximums at 420, 460, 490, 542, and 578 mμ, which indicate a moderate amount of hemoglobin, bilirubin, and carotene. It is interesting to note the similarity between chicken (curve 4) and cow plasma. Hemolysis in the chicken plasma is more pronounced than that for the horse and cow as indicated by the appearance of an absorption maximum at 542 mμ and an intensification of the absorption maximum at 578 mμ.

Figure 5 shows the absorption characteristics for the sheep plasma. The absorption maximums at 420, 542, and 578 mμ can be attributed to hemoglobin alone. The slight inflection point near 470 mμ would indicate a very small amount of bilirubin. No carotene is indicated. Visual inspection of the plasma occasionally showed a slight pink tinge indicating hemolysis. Hemolysis in the sheep plasma was more easily detected visually because of the lack of yellow color.

The absorption characteristics for the dog plasma (fig. 6) are very similar to those for the sheep plasma with absorption maximums at 420, 542, and 578 mμ with a slight inflection in the curve at 470 mμ. Thus, the absorption characteristics are due almost entirely to hemoglobin, while very little bilirubin is indicated, and apparently no carotene is present. The high general absorption characteristics exhibited in curve 3 were caused by suspended fat globules which are frequently found in plasma from carnivorous animals.

The cat plasma (fig. 7) is very similar to the dog and sheep plasma, both visually and in absorption characteristics. The more intense character of the 542- and 578-mμ absorption bands would indicate a greater degree of hemolysis.

*Heparin (Upjohn), 1,000 units (10 mg.) per cubic centimeter.

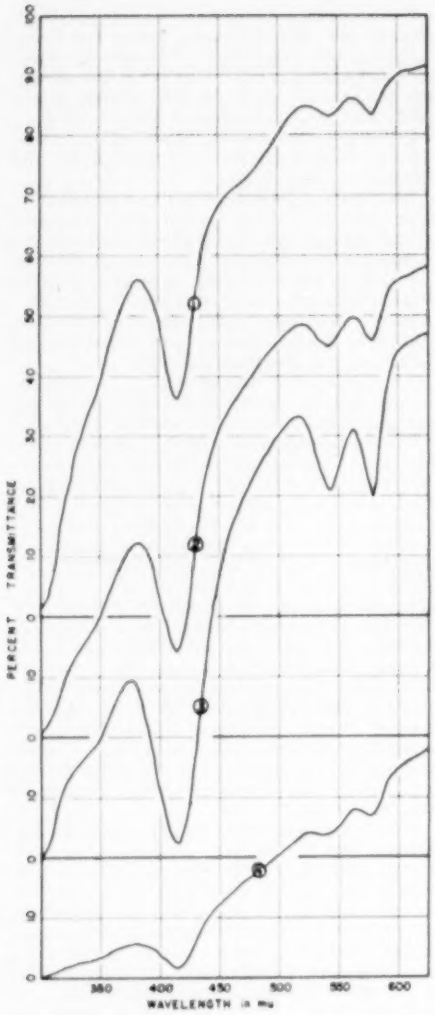

Fig. 7—Spectral transmittance curves for cat plasma.

Curve	Sampling date	Curve	Sampling date
1	Feb. 3, 1949	3	Feb. 17, 1949
2	Feb. 10, 1949	4	Feb. 24, 1949

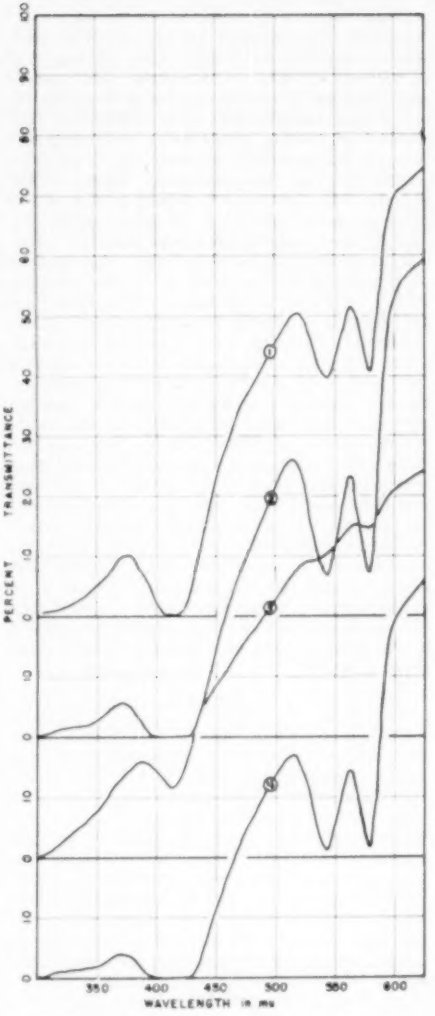

Fig. 8—Spectral transmittance curves for pig plasma.

Curve	Sampling date	Curve	Sampling date
1	Feb. 3, 1949	3	Feb. 17, 1949
2	Feb. 10, 1949	4	Feb. 24, 1949

The absorption spectrum for the pig plasma is shown in figure 8. Here again, as in the dog, cat, and sheep, the absorption characteristics can be attributed almost entirely to hemoglobin. The intense character of the 542- and 578-mμ absorption maximums indicates a higher degree of hemolysis in the pig than in any of the other animals studied. Hemolysis was quite evident visually.

Examination of the four weekly curves for the cow, horse, and sheep (fig. 3, 4, and 6, respectively) shows a remarkable uniformity in the absorption characteristics of the blood plasma for each animal. The absorption spectrums for the dog, cat, and pig (fig. 6, 7, and 8, respectively) show a variation in the degree of hemolysis over the period of observation. In addition, curve 3 for both the dog and the pig shows an anomalous behavior which is attributed to a turbid condition due to the presence of suspended fat globules.

SUMMARY

1) Absorption spectrums for plasma from a cow, horse, chicken, sheep, dog, cat, and pig at weekly intervals for four weeks are presented.

2) Qualitative analyses of these curves on the basis of hemoglobin, bilirubin, and carotene content have been accomplished.

3) Variations in absorption spectrums of plasma from individual animals over a limited period are shown.

4) Hemoglobin has been shown to be a component of plasma even when there is no visual evidence of its presence.

References

[1]Hoppe-Seyler, F.: Beitrage zur Kenntnis des Blutes des Menschen und der Wirbelthiere, Medicinisch-Chemische Untersuchungen, *3*, (1868): 366-385.

[2]Lewis, S. J.: The Ultra-Violet Absorption of Blood Sera, Proc. Roy. Soc., London, s. B., *89*, (1916): 327.

[3]Hoffman, W. S.: Photelometric Clinical Chemistry, Wm. Morrow and Co., New York, (1941): vii.

[4]Magath, T. B., and Sheard, C.: Spectrophotometric Analysis of Blood Serum in Normal and Pathological Conditions, Arch. Int. Med., *39*, (1927): 214.

[5]Drabkin, D. L., and Austin, J. H.: Spectrophotometric Studies. II. Preparation from Washed Blood Cells, Nitric Oxide Hemoglobin, and Sulfahemoglobin. III. Methemoglobin. IV. Hemochromogen. V. A Technique for the Analysis of Undiluted Blood and Concentrated Hemoglobin Solution. J. Biol. Chem., *112*, (1935): II, 51; III, 67; IV, 69; and V, 105.

[6]Davis, G. E., and Sheard, C.: Spectrophotometric Determination of Hemoglobin, Arch. Int. Med., *40*, (1927): 226.

[7]Sidwell, A. E., Jr., Munch, R. H., Barron, E. S. G., and Hogness, T. R.: The Salt Effect on the Hemoglobin-Oxygen Equilibrium, J. Biol. Chem., *123*, (1938): 335.

[8]Newcomer, H. S.: Absorption Spectra of Acid Hematin, Oxyhemoglobin, and Carbon-Monoxide Hemoglobin. A New Hemoglobinometer, J. Biol. Chem., *37*, (1919): 465.

[9]Anson, M. L., Barcroft, J., Mirsky, A. E., and Oinuma, S.: On the Correlation Between the Spectra of Various Haemoglobins and Their Relative Affinities for Oxygen and Carbon Monoxide, Proc. Roy Soc., London, s. B., *97*, (1925): 61.

[10]Fischer, Hans: Spectroscopic Examinations of Porphyrins and Hematin; Comment on Schumm's Correction. Ztschr. f. physiol. Chem., *155*, (1926): 96-98.

[11]Fischer, H., and Postowsky, J. J.: Determination of Active Hydrogen in Hemin and Bilirubin, Some of Their Derivatives and in Pyrrols. Ztschr. f. physiol. Chem., *152*, (1926): 300-312.

[12]Sheard, C., Baldes, E. J., Mann, F. C., and Bollman, J. L.: Spectrophotometric Determination of Bilirubin. Am. J. Physiol., *76*, (1926): 577.

[13]Sheard, C., Mann, F. C., and Bollman, J. L.: Spectrophotometric Determination of Purified Bilirubin. Am. J. Physiol., *81*, (1927): 774.

[14]Cutten, C., Emerson, E. E., and Woodruff, W.: The Icterus Index: Spectrophotometric and Quantitative Studies. Arch. Int. Med., *41*, (1928): 428.

[15]Muller, P., and Engel, L.: Uber das Absorptionsspektrum des Bilirubins in verschiedenen Lösungsmitteln. Ztschr. f. physiol. Chem., *202*, (1931): 56.

[16]Heilmeyer, L.: Das Absorptionsspektrum des Bilirubins in Chloroform, Alkohol und Alkalien. Biochem. Ztschr., *232*, (1931): 229.

[17]Smith, C. F.: The Ultra-Violet Absorption of Certain Aromatic Amino Acids and of the Serum Proteins. Proc. Roy. Soc., London, s. B., *104*, (1929): 198.

[18]Smith, C. F., and Marrack, J. R.: Further Observations on the Ultra-Violet Absorption Spectra of the Serum Proteins: The Specific Extinction Coefficients of Serum Pseudo-Globulin. Proc. Roy. Soc., London, s. B., *106*, (1930): 292.

[19]Sizer, I. W., and Peacock, A. C.: The Ultra-Violet Absorption of Serum Albumin and of Its Constituent Amino Acids as a Function of pH. J. Biol. Chem., *171*, (1947): 767.

[20]Carle, B. N., and Dewhirst, W. H.: A Method for Bleeding Swine, J.A.V.M.A., *101*, (1942): 495-496.

[21]Knacke, L. B.: Concerning Plasma Preparation in "Diseases of the Blood," Lippincott Co., Philadelphia, Pa., (1941): 571.

[22]Zechmeister, L.: Cis-Trans Isomerization and Stereochemistry of Carotenoids and Diphenylpolyenes. Chem. Rev., *34*, (1944): 296.

Studies of the Conglutinating Complement-Absorption Test. I. With Constant Complement

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INTEREST IN the conglutination test has recently been revived by a series of papers by Hole and Coombs.[1,2,3,4] In their first paper, these authors present a comprehensive review of the early literature and summarize the results of the few reports that have appeared on the subject during the past twenty years. As this review indicates, the conglutination test was used by the initial investigators in the diagnosis of typhoid, paratyphoid, dysentery, glanders, dourine, and several other diseases. The fact that in the intervening years it has been largely superseded by the hemolytic type of complement-fixation test would suggest that the conglutination method had proved less satisfactory and more difficult to standardize than the latter. However, on reinvestigating conglutination procedures, they have found[2] it possible to obtain reproducible results when all reagents are carefully controlled. On applying this quantitative technique, which they term the "conglutinating complement-absorption test" (CCA) to differentiate it from direct conglutination, in studies of experimental glanders in ponies, they found it to be more sensitive than the ordinary hemolytic test in detecting the presence of antibodies in serums from these animals. Furthermore, they demonstrate,[4] through the use of mallein and typhoid antiserums prepared in different animal species, that certain antiserums which do not fix guinea-pig (hemolytic) complement may exhibit definite fixation of the conglutinating complements from the horse and cat. These authors have been continuing the studies because they feel that the CCA test promises to be of definite value in the serum diagnosis of diseases in which hemolytic complement-fixation (HCF) tests have not given good results. The possibilities of the test in the diagnosis of Q fever rickettsial infection in cattle has been pointed out recently.[5]

The initial reports[1,2,3] suggested that the CCA test might be useful in some of our own problems, particularly in studies of the activities of certain avian antiserums that fail to fix hemolytic complement in the presence of homologous antigens,[6] for it seemed that this test might be simpler in application than the "indirect" complement-fixation test we had developed to meet this special need.[7,8] Moreover, at the time we were reinvestigating the usefulness of HCF in the standardization of different lots of mallein, we felt that conglutination might provide valuable supplementary information, particularly as results[3,4] had indicated that it might give a broader antigen-antibody coverage.

The first part of the present paper outlines in some detail the conglutination technique adopted as a result of our preliminary studies with mallein, Brucella, and Pneumococcus systems—a technique which is essentially that described by Hole and Coombs[2] with a few minor modifications. The second section presents a comparison of the antigen and antibody titers obtained in CCA tests with those determined by HCF tests.

TECHNIQUE OF CONGLUTINATING COMPLEMENT-ABSORPTION TEST

Like the HCF test, the CCA test is an indirect method of detecting and determining the approximate concentration of antigen or antibody in test mixtures as shown by the fixation or reduction in the activity of standardized amounts of complement. The two tests differ in the kinds of complement used and in the method of demonstrating the loss in complement activity. In the HCF test, the criterion of fixation of complement is a decrease in its hemolytic activity for sheep erythrocytes sensitized with antibody (amboceptor) usually prepared in rabbits. In the CCA test, the criterion of fixation is a decrease in the conglutinating activity of the complement for sheep red cells treated with heated bovine serum which supplies both sheep red cell antibody and conglutinin. Whereas, through the years, fresh guinea-pig serum has proved to be the most satisfactory source of hemolytic complement, that of the horse appears, on the whole, to be the most satisfactory for conglutination tests.[4]

The five reagents involved in the CCA test are antigen, antiserum, complement, sheep red cell suspension, and heated cow serum. Diluted antiserum, complement, and antigen are mixed and held for sixty minutes in a water bath at 25 C. The indicator system, 0.2 ml.

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of a mixture of equal parts of suitably diluted, heat-inactivated cow serum and 1 per cent sheep red cell suspension, is then added and the tests incubated for fifteen minutes in a 37 C. water bath. The mixtures are centrifuged at low speed for one minute and read by the resuspension technique. When no agglutination has occurred, the cells are readily resuspended. If agglutinated, the cells appear in irregular clumps that quickly settle out leaving a clear or somewhat cloudy supernatant. Readings are recorded as 0 to 4+ depending upon the degree of agglutination and the relative clarity of the supernatant.

Complement.—Blood is collected from a healthy horse, allowed to clot, and the serum taken off and centrifuged. Any excess of serum over that needed for the day's test may be frozen immediately, since horse complement maintains its titer for weeks in the frozen state but deteriorates rapidly at room temperature or in the refrigerator.

A 1 : 40 or 1 : 80 dilution of complement is prepared and the following quantities pipetted in duplicate to the bottom of 11 by 75 mm. tubes: 0.10, 0.09, 0.08, 0.07, 0.06, 0.05, 0.04, 0.03, 0.02, and 0.01 ml. Sufficient 0.85 per cent sodium chloride solution is added to bring the volume to 0.3 ml. in each tube; the mixtures are shaken, and 0.2 ml. of the sensitized sheep red cells added. After fifteen minutes' incubation at 37 C., the mixtures are centrifuged and the reactions read in the usual manner. The smallest amount of complement showing complete clumping of the sensitized sheep red cells (4+) is taken as one unit. Dilutions of complement are prepared to contain two units in 0.1 ml.; these dilutions should be kept in a pan of ice water during use. If the time of filling is prolonged beyond one hour, fresh dilutions of horse complement should be made.

Bleedings collected at different times from the same horse may vary in complement titer.

Indicator Mixture.—The indicator mixture is prepared by adding diluted, heated bovine serum to an equal volume of a 1 per cent suspension of sheep red cells.

Sheep Red Cells.—Well-washed, fresh or preserved sheep red cells are made up to a 1 per cent suspension, the turbidity of which is checked electrometrically.

Bovine Serum.—Fresh bovine serum is heated for thirty minutes at 56 C. and maintained in the frozen state until required. In determining its titer, serial twofold dilutions from 1 : 5 to 1 : 80 are prepared. To each serum dilution is added 0.1 ml. of the two-unit dilution of horse complement, 0.2 ml. of salt solution, and 0.1 ml. of a 1 per cent sheep red cell suspension. The tests are well shaken, incubated for fifteen minutes at 37 C., centrifuged, and read. The highest dilution of cow serum inducing complete conglutination is noted and dilutions prepared to contain twice this amount in 0.1 ml. (or in 0.05 ml. for tests to which diluted rabbit amboceptor is added as an antibody supplement).

The titers of the two essentials contributed by the cow serum, antibody for sheep red cells and conglutinin, rarely parallel each other[5] in serums of individual animals. One of these variables may be eliminated by adding a very small amount of sheep red cell amboceptor to cow serum of low sheep red cell antibody titer; this is not required with cow serums of average or high titer. Care must be taken not to add enough to bring about the agglutination of the red cells in the absence of complement.

The amount of rabbit amboceptor added has ranged from one eighth to one fifth of the quantity used in HCF tests to maximally sensitize the 5 per cent sheep red cell suspension. Dilutions are prepared to contain this amount in 0.05 ml. Equal volumes of diluted amboceptor and the diluted heated cow serum are mixed. After standing for about five minutes, this mixture is added to an equal volume of 1 per cent sheep red cell suspension.

Standard Antigen and Antiserum.—The same general principles govern the standard-

TABLE I—Conglutinating Complement-Absorption and Hemolytic Complement-Fixation Serum-Dilution Titers of Serial Bleedings from 6 Horses Injected with Malleins Prepared on Broth or Synthetic Medium

Horse* (No.)	Date of bleeding (1948)	Titer** CCA	Titer** HCF	Horse* (No.)	Date of bleeding (1948)	Titer** CCA	Titer** HCF
1	10/22	1 : 2	1 : 2	4	10/22	<1 : 2	1 : 2
	10/29	1 : 2,000	1 : 1,000		10/29	1 : 500	1 : 200
	11/5	1 : 500	1 : 500		11/5	1 : 100	1 : 200
2	10/22	1 : 2	1 : 5	5	10/22	<1 : 2	1 : 2
	10/29	1 : 5,000	1 : 5,000		10/29	1 : 20	1 : 50
	11/5	1 : 2,000	1 : 5,000		11/5	1 : 100	1 : 50
3	10/22	1 : 5	1 : 2	6	10/22	1 : 2	1 : 5
	10/29	1 : 2,000	1 : 2,000		10/29	1 : 100	1 : 100
	11/5	1 : 1,000	1 : 2,000		11/5	1 : 100	1 : 100

*Horses 1, 2, and 3 received broth mallein; horses 4, 5, and 6, synthetic mallein. **Synthetic mallein, XXII, was used as antigen in both tests.

ization of antigens and antiserums for the CCA tests as for the HCF tests.

Antigen.—All antigens are first tested undiluted and in serial twofold dilutions for anticomplementary activity with one unit of complement. Serial twofold dilutions of antigen, beginning with one quarter of the minimum and anticomplementary dose, are set up with the "standard" dilution of antiserum, or, in preliminary tests, with a dilution of antiserum containing sufficient antibody to fix with homologous antigen, twice the amount of complement added. If too great an excess of antibody is used, prozone tendencies may be obscured.

Twice the smallest average quantity of antigen with which complete inhibition of conglutination is observed in repeated titrations with two units of complement, is taken as the standard amount for such tests.

Antiserum.—Serial dilutions of antiserum are mixed with two units of complement and the standard dilution of antigen (or in preliminary titrations with ¼ of the minimum anticomplementary dose) and incubated for the hitherto stated periods at 25 and 37 C. Controls of the anticomplementary activities of these serum dilutions accompany the test.

The quantity of antiserum selected as standard is twice the minimum required for complete inhibition of fixation in repeated titrations with two units of horse complement; this quantity must have no appreciable anticomplementary effect.

Tests for Antibody.—In testing a serum for the presence of antibody, serial twofold (or closer) dilutions are prepared. To 0.1-ml. amounts of these dilutions are added 0.1 ml. of the two-unit dilution of horse serum and 0.1 ml. of the standard dilution of antigen. After sixty minutes incubation at 25 C., 0.2 ml. of sensitized sheep red cells is added, the mixtures held in the 37 C. water bath for fifteen minutes, centrifuged, and read. Antigen controls as well as controls of the effect on complement of 0.1 ml. of the first three or six dilutions of each test serum (without antigen) are included in all titrations.

Since serums of many animals contain natural agglutinins for sheep red cells, it is also advisable to test the lower dilutions of each serum for agglutinative activity for sensitized sheep red cells in the absence of complement. Serums found to have a high agglutinative titer should be absorbed with sheep red cells before retesting by the CCA method.

The titer of the antiserum is expressed as the highest dilution that completely inhibits conglutination and is without anticomplementary or agglutinative activity. When a comparable degree of inhibition of conglutination is observed in tests with and without antigen, the reaction is classified as anticomplementary.

Tests for Antigen.—Serial twofold dilutions of the material to be tested for the presence of antigen are prepared and pipetted in 0.1-ml. amounts in duplicate. To the first set, 0.1 ml. of the two-unit dilution of complement and 0.1 ml. of salt solution is added; to the second set, 0.1 ml. of the two-unit complement dilution and 0.1 ml. of the standard dilution of antiserum. The first set, therefore, serves as a control of the anticomplementary properties, the second, of the antigenic activity of the test material. The antigenic titer is expressed in terms of the highest dilution of antigen, without significant anticomplementary properties, that produces complete inhibition of conglutination in the presence of antiserum.

Complement controls (0.1 ml. of the two-unit dilution of complement and 0.2 ml. of salt solution) as well as controls of the stability of the sensitized sheep red cell suspension are included in both the antigen and the antibody tests. Complete conglutination should be recorded in the former, none in the latter.

It will be evident from the foregoing, that only minor modifications have been introduced in the CCA methods of Hole and Coombs.[5] These were a reduction of the total volume of the test from 2.0 to 0.5 ml., the use of a 1 per cent rather than an 0.5 per cent suspension of sheep red cells, preliminary incubation at 25 C., and secondary incubation at 37 C. rather than at room temperature throughout, and, when required, the addition of a very small amount of sheep red cell amboceptor to supplement the natural antibody in the heated cow serum.

Technique of Hemolytic Complement-Fixa-

TABLE 2—Conglutinating Complement-Absorption and Hemolytic Complement-Fixation Antigen-Dilution Titers of Different Synthetic Mallein Antigens in Tests with Horse and Rabbit Antiserums

Preparation	Culture strain	Period of incubation (months)	Antiserum			
			Horse		Rabbit	
			CCA	HCF	CCA	HCF
XXII	325A, 326A$_1$	3	1 : 2,560	1 : 1,280	1 : 2,560	1 : 1,280
XXIV a	325A	2	1 : 20	1 : 40	1 : 40	1 : 80
XXIV c	325A	3	1 : 320	1 : 160	1 : 320	1 : 160
XXIV b	326A$_1$	2	1 : 160	1 : 1,280	1 : 320	1 : 640
XXIV d	326A$_1$	3	1 : 320	1 : 640	1 : 640	1 : 640

tion Tests.—Antibody titers determined by the foregoing method were compared with those obtained in HCF tests of the serum-dilution type. In this method, 0.1 ml. of serial dilutions of serum were titrated with a constant dilution of complement (0.1 ml. of a dilution containing three or four 50 per cent hemolytic units) and 0.1 ml. of a standard dilution of antigen. After a period of preliminary incubation of ninety minutes at 37 C., 0.2 ml. of a mixture of a 5 per cent sheep red cell suspension with an equal volume of suitably diluted sheep red cell amboceptor was added. The period of secondary incubation was thirty minutes at 37 C. The reagents for this test were prepared as described in "Standard Methods of the Division of Laboratories and Research of the New York State Department of Health."[9]

EXPERIMENTAL RESULTS

As has been stated earlier, we had a twofold purpose in taking up the study of the CCA test: (a) to determine whether it would be more sensitive than HCF methods in the titration of antigen-antibody mixtures capable of reacting with both hemolytic and conglutinating complements, and (b) to ascertain whether this test would detect activity in certain antigen-antibody systems that had consistently failed to fix guinea-pig (hemolytic) complement.

Relative Titers of Conglutinating Complement-Absorption and Hemolytic Complement-Fixation Tests.—Three antigen-antibody systems were used in comparing the relative sensitivity of CCA and HCF methods—mallein, Brucella, and Pneumococcus. The data summarized here will be presented in greater detail elsewhere.[10,12]

Mallein Systems.—Bleedings collected at periodic intervals from horses and rabbits under immunization with mallein prepared on broth or synthetic medium were tested in parallel in CCA and HCF tests of the serum-dilution type. Three 50 per cent hemolytic units were added in the HCF tests, two complete conglutinating units in the CCA tests. The former amount is less than the two complete hemolytic units previously used[3] in comparative studies, but represents the level of sensitivity we had previously adopted as satisfactory for mallein HCF tests.[2]

Table 1 illustrates the general agreement between the CCA and HCF titers of three bleedings from each of 6 horses. The first bleeding was collected Oct. 22, 1948, after the horses had been rested for three months following an earlier series of subcutaneous injections of broth (horses 1, 2, and 3), or synthetic mallein (horses 4, 5, and 6). The first recall dose of mallein was given intravenuously on the same day, a second injection one week later, October 29, immediately after a second bleeding. The horses were bled again on November 5. The October 22 bleedings displayed little complement-fixing activity in either CCA or HCF tests. The second and third bleedings showed a marked rise in titer, particularly those from horses receiving broth mallein. Five of these 12 postinjection bleedings had the same titers in CCA and HCF tests; three had higher titers in the former and four in the latter. In no case was the difference greater than twofold, a difference which is not statistically significant.

Periodic bleedings from 2 other horses that had been immunized with another lot of synthetic mallein showed a similar degree of agreement between titers determined by CCA and HCF methods.

Our failure to demonstrate significant differences in the relative complement-fixing activities of mallein horse antiserums with guinea-pig and horse complement seems to be in some disagreement with earlier results,[3] when the CCA test was found to be more sensitive than the HCF test in detecting the presence of antibodies in the serums of infected ponies. However, since our serums were prepared by the intravenous and subcutaneous injection of mallein filtrates, it seems conceivable that the resultant antibodies may have differed from those induced by infection in the antigenic elements toward which they were directed, or possibly even in the serum globulin com-

TABLE 3—Conglutinating Complement-Absorption and Hemolytic Complement-Fixation Titers with *Brucella Abortus* Suspension of 62 Bovine Serums Reported as Reactive in Routine Diagnostic Agglutination Tests

CCA titer	No. with HCF titer of:									
	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320	1 : 640	1 : 1,280	1 : 2,560	1 : 5,120	Total
1 : 160	1	2								3
1 : 320		1								1
1 : 640		2	3	3	1					9
1 : 1,280			2	3	4					9
1 : 2,560				1	5	3				9
1 : 5,120					1	6	6			13
1 : 10,240						2	2	9	5	18
Total	1	5	5	7	11	11	8	9	5	62

ponent with which they were associated. Such differences in the nature of the respective antibodies, if existent, might be reflected in their relative behaviour with horse and guinea-pig complement in the presence of mallein antigen.

The CCA and HCF tests were also used in parallel in testing the relative activities of a series of malleins prepared with two different strains of *Malleomyces mallei*, 325A and $326A_1$, on broth and synthetic medium and harvested after two or three months' incubation. Mallein antiserums from both horses and rabbits were employed in these titrations. The results for five representative lots of mallein are given in table 2.

In tests with both horse and rabbit antiserums, synthetic mallein XXII had a titer of 1 : 2,560 in CCA as compared with 1 : 1,280 in HCF tests. Lower dilutions of this mallein were somewhat anticomplementary in the former test. Both tests indicated a definite rise in antigenic titer in the strain 325A cultures during the third month of incubation. According to the HCF test with horse antiserum, the strain $326A_1$ preparation harvested at two months had a higher titer than that harvested at three months; a more precise quantitative technique with varying amounts of complement verified the fact that these two preparations differed in complement-fixing properties. In HCF tests with rabbit antiserum, however, these two lots of $326A_1$ mallein had the same titer. The CCA titers of the two months' culture filtrate were higher than the HCF titers of the same; the CCA and HCF titers of the three months' filtrate were comparable.

Whether the differences in complement-fixing activities shown by these and various other malleins with horse and rabbit antiserums and in the two forms of titration are indicative of actual variation in antigenic make-up remains to be shown. Since the question is important from the standpoint of mallein standardization, it is receiving further attention.

Brucella Systems.—In extending these comparative studies of the two types of complement fixation to titrations with a particulate antigen and to antiserum from another animal species, a small group of 134 specimens of bovine serums, submitted for examination for evidence of brucellosis were tested in parallel by the CCA and HCF techniques, using suitable dilutions of a *Brucella abortus* suspension.

Of the 134 serums, 62 had been reported as positive in the two routine diagnostic agglutination procedures (slide and tube methods). All of these were also reactive in both complement-fixation tests. As is indicated in table 3, the titers were always higher in CCA than in HCF tests. The value of the ratio of the CCA to the HCF titer was 2 for 6, 4 for 31, 8 for 16, and 16 for 9 serums.

The remaining 72 specimens had been negative in the diagnostic agglutination tests with *Br. abortus* antigen. Of these, 48 did not fix complement with this antigen in either the CCA or HCF; 16 were negative in HCF but anticomplementary in CCA tests. None of the specimens was anticomplementary in both tests. The anticomplementary properties toward horse complement increased more rapidly on storage than those toward guinea-pig complement.

In short, therefore, although the CCA test proved to be more sensitive than the HCF technique in the titration of positive bovine serums, a larger proportion were found to be anticomplementary. Since, in the presence of antigen, this zone of nonspecific fixation may extend one or two tubes beyond the anticomplementary range, it is more difficult to interpret the significance of reactions in the CCA test.

Pneumococcus Systems.—Pneumococcus type 1 and type 2 horse antiserums failed to fix horse complement in the presence of homologous type-specific carbohydrate, just as they have been repeatedly shown to lack complement-fixing activity with the same antigens in HCF tests.[13] On the other hand, mixtures of Pneumococcus rabbit antiserums with homologous carbohydrate antigen readily fixed both guinea-pig and horse complement.

In CCA titrations with a constant amount of type 1 Pneumococcus rabbit antiserum and serial dilutions of type 1 carbohydrate, a prozone was noted with an excess of antigen, an intermediate zone of complete fixation, and a postzone of partial, then of negative, reactions. The zone of complete fixation, although somewhat broader than that observed in HCF tests, roughly coincided with the latter. Similar results were obtained with type 3 Pneumococcus rabbit antiserums and type 3 carbohydrate.

In parallel titrations of a small number of types 1, 2, and 3 Pneumococcus rabbit antiserums with suitable dilutions of homologous type-specific carbohydrate, the CCA titers were similar to, or in some cases slightly higher than, the HCF titers.

These Pneumococcus systems have been used in studying the quantitative relationships between each of the reagents in CCA tests.[12]

Avian Antiserums.—A practical difficulty in the assay of the antibody content of chicken, turkey, and various other avian

antiserums has been their failure to fix guinea-pig complement in the presence of homologous antigen. Where other serologic methods, such as agglutination or precipitation, are applicable, this lack of complement-fixing activity is not of much practical importance. In means, however, that the hemolytic complement-fixation methods, which have proved so valuable in investigation of viral infection in mammalian species, are not available for studies of such infections in these birds. Although the indirect complement-fixation test[7] can be used in titrating viral avian antiserums,[14,15] one is always confronted with the problem of obtaining a satisfactory complement-fixing antiserum from some other animal species for the secondary system. Since a direct test is obviously simpler, a search was undertaken for some other type of complement, hemolytic or conglutinating, which would be fixed by such avian antibody-antigen systems.

Preliminary studies with horse complement have given negative results in the following systems: chicken and turkey *pullorum* antiserums with *Salmonella pullorum* suspensions, Newcastle disease virus antigen with serums of chickens convalescent from infection with this agent, and influenza virus chicken antiserums with allantoic fluids from chicken embryos inoculated with this virus. Other serologic methods, agglutination in the case of the first system and hemagglutination-inhibition with the second and third, indicated the presence of high antibody titers in these various chicken antiserums.

DISCUSSION

These preliminary studies of the CCA reaction, in agreement with earlier findings,[3,4] have shown that under carefully controlled conditions this test may provide results in relatively good agreement with those obtained by the hemolytic complement-fixation technique, or, in some instances, may prove definitely more sensitive than the latter method in detecting the presence of antigen-antibody combinations. The conglutination reaction is relatively easy to read but does not permit differentiation of finer degrees of reaction. It cannot be expected, therefore, to lend itself to the more precise quantitative estimation of antigen and antibody content that is possible with the HCF test. However, where a test of the serum- or antigen-dilution type with constant complement is considered to provide sufficiently accurate information, the two tests may be about equally satisfactory. In the case of bovine antiserums, or serums of various other animal species,[4] the greater sensitivity of the CCA method may indeed outweigh in importance its lesser precision, provided of course this gain in sensitivity has not been accompanied by a proportionate loss in specificity. It would seem well worthwhile to reinvestigate a number of antigen-antibody systems that have given unsatisfactory results in HCF tests with guinea-pig complement, to determine whether the CCA method with horse or with other conglutinating complements might be of practical value.

The observation that certain serums may be anticomplementary with guinea-pig but not with horse complement, or vice versa, may also have practical significance. It suggests that in dealing with anticomplementary specimens, always a problem in diagnostic work, that CCA might function as a useful supplement to HCF tests.

SUMMARY

The conglutinating complement-absorption (CCA) technique,[2] with minor modifications, has been applied in parallel with a hemolytic complement-fixation (HCF) test of the serum-dilution type in preliminary studies of a number of different antigen-antibody systems.

In the titration of mallein antiserums prepared in horses and rabbits by subcutaneous injection of mallein produced on broth or synthetic medium, the two tests appeared to possess relatively comparable sensitivity. Pneumococcus rabbit antiserums in mixture with homologous type specific carbohydrate, also fixed guinea-pig and horse complement to a relatively comparable degree. In tests of bovine serums with *Brucella abortus* suspension, however, the CCA test was more sensitive than the HCF method employed. Pneumococcus horse antiserum and various chicken antiserums, which had been previously shown to lack the capacity for fixing guinea-pig complement in the presence of homologous antigen, also failed to fix horse complement with the same antigens.

Some of the possible practical applications of the CCA test are discussed briefly.

References

[1]Hole, N. H., and Coombs, R. R. A.: The Conglutination Phenomenon. I. An Introduction to the Conglutination Phenomenon and an Account of the Observations and Views of Previous Investigators. J. Hyg., 45, (1947):480-489.

[2]Hole, N. H., and Coombs, R. R. A.: The Conglutination Phenomenon. II. The Technique of the Conglutinating Complement Absorption Test Compared with the Hemolytic Complement Fixation Test. J. Hyg., 45, (1947):490-496.

[3]Hole, N. H., and Coombs, R. R. A.: The Conglutination Phenomenon. III. The Conglutinating Complement Absorption Test in Experimental Glanders. J. Hyg., 45, (1947):497-503.

[4]Coombs, R. R. A., and Hole, N. H.: The Con-

glutination Phenomenon. IV. The Importance of the Choice of Complement when Examining Antisera for the Presence of Complement-Fixing or Complement-Absorbing Antibodies. J. Hyg., *46*, (1948): 296-310.

[8]Wolfe, D. M., and Kornfeld, L.: Conglutinating Complement Absorption Test Compared with Hemolytic Complement-Fixation Reactions Using Q Fever Immune Bovine Serum. Proc. Soc. Exptl. Biol. and Med., *69*, (1948):251-255.

[9]Rice, C. E.: Atypical Behaviour of Certain Avian Antisera in Complement-Fixation Tests. Canad. J. Comp. Med. and Vet. Sci., *11*, (1947):236-245.

[10]Rice, C. E.: Some Factors Influencing the Selection of a Complement-Fixation Method. II. Parallel Use of the Direct and Indirect Technics. J. Immunol., *60*, (1948):11-16.

[11]Rice, C. E., and Gwatkin, R.: Studies in Pullorum Disease. XXIV. A Comparison of Titers Obtained by Indirect Complement-Fixation and Agglutination Methods for Chicken Sera. Canad. J. Comp. Med. and Vet. Sci., *10*, (1949):165-172.

[12]Wadsworth, A. B.: Standard Methods of the Division of Laboratories and Research of the New York State Department of Health, Albany, N. Y., Williams and Wilkin Co., Baltimore, Md. 3rd ed., (1947):361-465.

[13]Rice, C. E., McIntosh, C. W., Konst, H., and Duthie, R. C.: Complement-Fixation in the Standardization of Mallein. Canad. J. Comp. Med. and Vet. Sci. To be published.

[14]Rice, C. E.: Studies of the Conglutinating Complement-Absorption Test. II. With Varying Amounts of Complement. Canad. J. Comp. Med. and Vet. Sci. In press.

[15]Rice, C. E., and Tallyour, J.: Application of Conglutinating Complement-Absorption Test in the Titration of Bovine Sera with *Brucella Abortus* Antigens. Canad. J. Comp. Med. and Vet. Sci. To be published.

[16]Zinsser, H., and Parker, J. T.: Observation on a Substance in Immune Horse Serum Which Interferes with Alexin Fixation. J. Immunol., *8*, (1923): 151-161.

[17]Wolfe, D. M., Kornfeld, L., and Markham, F. S.: Simplified Indirect Complement-Fixation Test Applied to Newcastle Disease Immune Avian Serum. Proc. Soc. Exptl. Biol. and Med., *70*, (1949):490-494.

[18]Hilleman, M. R., and Helmold, R. J.: Indirect Complement-Fixation Test for Psittacosis-Lymphogranuloma Venereum Group Antibodies in Chicken Antisera. Federation Proc., *8*, (1949):405.

The Effect of a Thiamine Analogue on the Histopathology of Rat Tissues

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IN A FORMER PAPER,[1] the effects of a thiamine analogue, *o*-aminobenzyl-(3)-4-methyl-5-β-hydroxyethylthiazolium chloride hydrochloride,* on growth and on the thiamine content of rat tissues were reported. It was found that growth was inhibited slightly in weanling rats by feeding this analogue. Clinically, the analogue did not produce ill effects, as the rats receiving the analogue could not be differentiated from those in the control groups. The thiamine concentration in the liver, kidney, and skeletal muscle was slightly lower when the analogue was fed but the difference was not significant.

This paper includes the observed histopathologic changes occurring in selected tissues. Sciatic nerves were examined with a polarizing microscope. Tissues stained with hematoxylin and triosin were compared with those stained by the Marchi method.

LITERATURE

Various methods have been used in studying histologic changes occurring in tissues. As early as 1854, Virchow and other investigators used the polarizing microscope in studying nerve tissue. They noticed that myelin sheaths of nerve fibers were optically active.

Polarized light is known to vibrate in one plane only, while ordinary light vibrates in all directions perpendicular to the direction in which it travels. Substances appearing black between crossed prisms of the polarizing microscope are isotropic. When substances appear light under the same conditions, they are said to be anisotropic or show the property of birefringence.

Myelin, a mixture of compound lipids, changes from its normal anisotropic appearance to an isotropic state when it undergoes degeneration. The exact chemical change appears to be unknown. Sutton and coworkers[2] realized that myelin may change to triglycerides, which are isotropic, thus permitting a more accurate study of myelin sheath degeneration with the polarizing microscope. Baird[3] states that one and one-half hours after a myelinated nerve is severed, degeneration can be observed with a polarizing microscope. After three hours, the fibers are swollen and the myelin has separated into masses.

Sutton and coworkers[2] produced myelin sheath degeneration of peripheral nerves in rats by feeding a vitamin A-deficient diet. Lee and Sure[4] obtained results similar to those of Sutton and coworkers. They produced myelin sheath degeneration in the sciatic nerves of rats by feeding thiamine-deficient, vitamin A-deficient, and B-complex-deficient diets.

Prickett and coworkers[5] demonstrated a marked difference in the degree of myelin sheath degeneration of rats by varying the thiamine intake. In acute deficiencies, the appearance of the nerves was essentially normal, while rats receiving a suboptimal level of thiamine for a longer period of time showed a considerable amount of myelin sheath degeneration. Control groups, receiving an adequate amount of thiamine over a long period but limited in food intake, showed more degeneration than thiamine-deficient rats.

The transformation of normal myelin to its degenerated state can also be shown by fat stains. Hassin[6] states that, twenty-four hours after a nerve is severed, the Marchi staining method shows that the myelin has become swollen, irregular, and separated into fragments. Degenerated myelin becomes black on exposure to osmic acid in this method of staining. Setterfield and Sutton[7] observed black droplets in the nerves of thiamine-deficient rats by using the Marchi stain. This finding checked closely with their polarized light study.

Davison and Stone[8] observed as many lesions in the nervous system of starved rats as those receiving diets deficient in thiamine or riboflavin. The lesions were vacuolation, liquefaction necrosis, and chromatolysis of the ganglion cells of the brain and spinal cord. The myelin sheaths of the peripheral nerves were also degenerated.

Shaw and Phillips[9] observed in acute thiamine deficiency a small amount of myelin sheath degeneration in the peripheral nerves and spinal cords of 6- to 8-week-old pigeons, while the peripheral nerves and spinal cords

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*The thiamine analogue, *o*-aminobenzyl-(3)-4-methyl-5-β-hydroxyethylthiazolium chloride hydrochloride, was supplied by R. R. Sealock, Ph.D., associate professor of chemistry at Iowa State College.

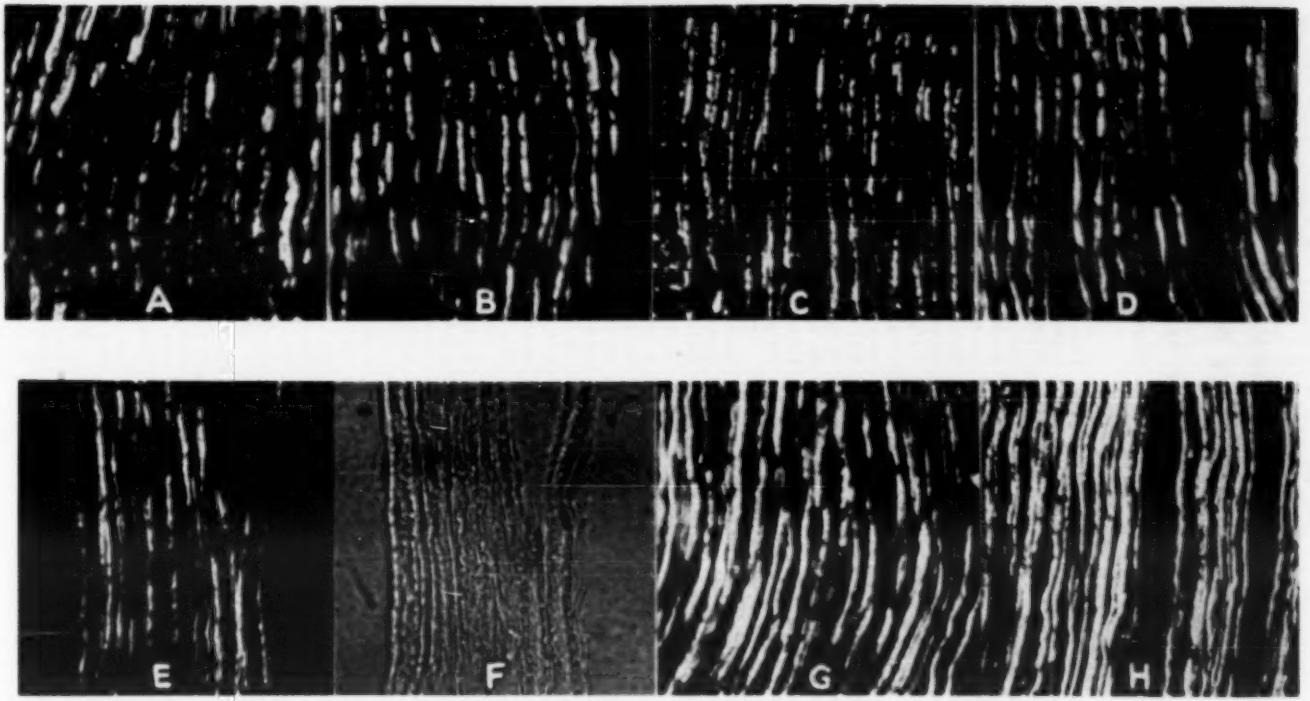

Fig. 1—Photomicrographs of sciatic nerves showing myelin sheath degeneration. All the pictures were taken between crossed prisms of a polarizing microscope except "f," which was taken between uncrossed prisms. x 500.

of day-old chicks appeared normal. Symptoms of acute thiamine deficiency in the young, growing chicks appeared in eight to nine days, while a longer time was required for the pigeons, which accounted for the difference in the myelin sheath degeneration. Chronic thia-

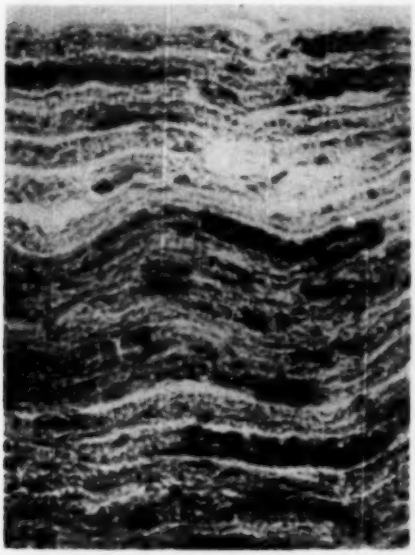

Fig. 2—Longitudinal section of sciatic nerve from rat 108 showing myelin sheath degeneration. Marchi. x 500.

mine deficiency produced a moderate amount of degeneration in the myelin sheaths of both pigeons and chicks.

Phillips and Engel[11] demonstrated with polarized light and by the Marchi method of staining that a riboflavin deficiency in chicks caused histologic changes characterized by degeneration of the myelin sheaths in the peripheral nerves and the spinal cord. Muscle fibers were also degenerated.

Engel and Phillips[11] believe that rats receiving a balanced diet, with the exception of thiamine, seldom show pathologic changes in the peripheral nerves, spinal cord, brain, and kidneys. When thiamine was given to thiamine-deficient rats, fatty degeneration of the liver occurred. The nuclei of the liver cells remained normal.

Eveleth and Biester[12] found that severe myelin sheath degeneration in swine was not the cause of incoördination. The animals recovered from the incoördination following the supplementation of vitamin A and green feeds, but the myelin sheath degeneration was still present. Vitamin A and the B-complex vitamins were eliminated as possibilities in causing incoördination and myelin sheath degeneration in swine. Control pigs on pasture did not exhibit myelin sheath degeneration in the spinal cord. It is presumed that the animals on pasture received some nutritional factor which was absent in the diets of those showing degeneration in the spinal cord.

Evans, Carlson, and Green[13,14] found the primary lesions in Chastek paralysis to be in the liver, heart, and brain. The liver showed severe fatty degeneration, congestion, hemorrhages, and necrosis of the hepatic cords. Focal necrosis appeared in the heart along with hemorrhages, cloudy swelling, enlargement of the nucleoli in the myocardial cells, and proliferation of connective tissue.

Vedder[15] states that in the early stages of beriberi, the heart becomes hypertrophied, followed by dilatation and cardiac failure. There is chronic passive congestion of the liver, spleen, kidneys, and intestines. Degenerative changes are present in the liver. The skeletal muscles become atrophied with a loss of striations, shrinkage of sarcoplasm, and degenera-

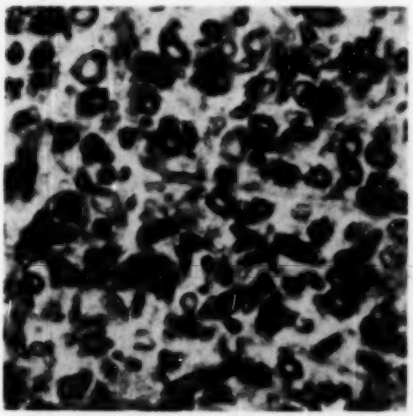

Fig. 3—Cross section of spinal cord from rat 108 showing myelin sheath degeneration. Marchi. x 500.

tion. The myelin sheaths in the peripheral nerves and spinal cord are degenerated. Engel and Phillips[11] believe that in uncomplicated cases of beriberi, myelin sheath degeneration does not occur.

Methods and Results

This investigation was designed to determine what effect a thiamine analogue, *o*-aminobenzyl-(3)-4-methyl-5-β-hydroxyethylthiazolium chloride hydrochloride, may have on the various rat tissues. A study comparing the histopathologic changes in rats receiving the

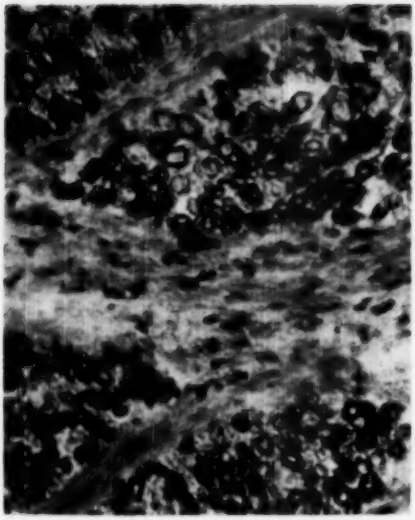

Fig. 4—Brain medulla of rat 112 showing myelin sheath degeneration. Marchi. x 500.

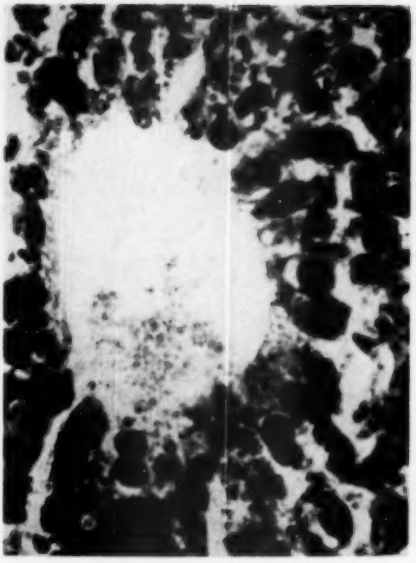

Fig. 5—Liver of rat 124 showing a fatty change around a central vein. Marchi. x 300.

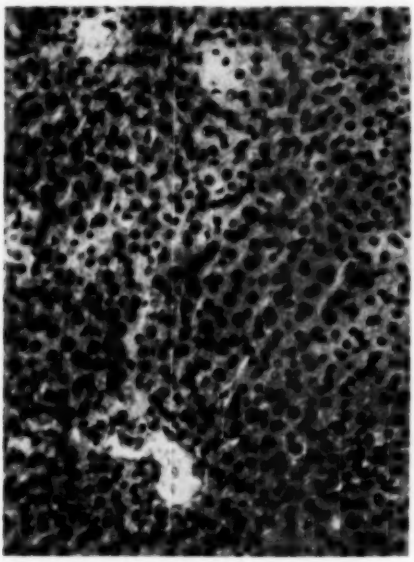

Fig. 6—Liver of rat 108 showing atrophy. Note number of nuclei per unit of space. Hematoxylin-triosin. x 300.

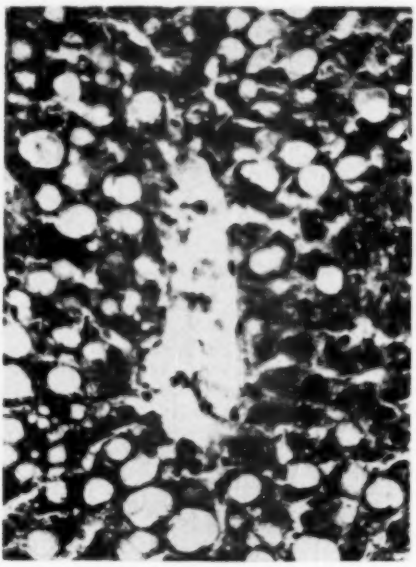

Fig. 7—Liver of rat 109 showing a fatty change around a central vein. Hematoxylin-triosin. x 300.

analogue with their control groups has been made.

Weanling rats, 4 weeks old, were divided into six groups. Groups A through E received a basal diet *ad lib.*, which was void of thiamine. Group F received a commercial fox chow *ad lib.* Some of the groups received supplements as shown in table 1. A description of the experimental procedure, including the contents of the basal diet and the preparation of the supplements, has been described in a previous paper.[1]

The rats in groups A and D, receiving the thiamine-free diet and apparently affected with acute thiamine deficiency, showed a considerable amount of dehydration and atrophy of the skeletal muscles. It was particularly noticeable in rat 108, which had lost 15 Gm. from its starting weight. The rats receiving the thiamine-free diet consumed less basal diet and water than those receiving thiamine-deficient diets. They were also observed as being less active than those receiving thiamine in their diet. The hearts from the rats receiving the thiamine-free diet were atonied. Hydropericardium was present in the rats of series 1, while it was not observed in series 2. Grossly, the remaining tissues appeared normal.

A portion of sciatic nerve from each rat of series 1 was placed in 10 per cent formol-saline solution immediately after chloroform euthanasia. On the following day, longitudinal sections, 10 μ in thickness, were made of the nerves with a freezing microtome. The sections were floated in water, placed on a clean slide, mounted in glycerin, and covered with a clean cover glass. At this time, the sections were examined between crossed prisms of a polarizing microscope. Photomicrographs were taken of the nerves at the point of greatest birefringence.

TABLE 1—Comparison of Growth Data from Two Series of Rats Receiving the Thiamine-Free Basal Diet* with Daily Supplements of Thiamine and the *o*-Aminobenzyl Analogue**

Rat No.		Daily supplementation of B_1 and its *o*-aminobenzyl analogue	Days on expt.	Wt. when killed (Gm.)	Gain or loss (Gm.)
Series 1	Series 2				
Group A		0			
108			35	46	—15
110			38	48	— 8
109		(5 μg. B_1 last 32 days)	72	102	40
111		(5 μg. B_1 last 32 days)	72	95	37
	145		16	58	2
	147		16	46	— 2
Group B		5 μg. B_1			
112			36	108	40
114			38	64	4
113		(5 add. μg. B_1 last 16 days)	59	123	57
115		(5 add. μg. B_1 last 20 days)	63	161	99
	149		30	88	32
	150		33	106	50
	151		35	106	56
	152		37	98	44
Group C		5 μg. B_1 and 1 mg. B_1 analogue			
116			38	106	40
118			36	64	4
117		(5 add. μg. B_1 last 16 days)	59	123	57
119		(5 add. μg. B_1 last 20 days)	63	161	99
	153		30	88	32
	154		33	106	50
	155		35	106	56
	156		37	98	44
Group D		1 mg. B_1 analogue			
121			38	66	— 2
122			36	57	— 7
120		(5 μg. B_1 last 29 days)	70	104	38
123		(5 μg. B_1 last 29 days)	70	119	53
	157		22	52	— 5
	158		17	59	2
	159		17	64	12
	160		23	60	4
Group E		5 μg. B_1			
124			42	161	107
125			66	217	157
126			66	164	104
	162		38	204	151
	163		38	115	70
	164		38	203	156
Group F		0			
127			77	278	193
128			46	281	199

*Group F received a commercial fox chow *ad lib.* **o-aminobenzyl-(3)-4-methyl-5-β-hydroxyethyl-thiazolium chloride hydrochloride.

Figure 1 consists of several photomicrographs of the sciatic nerve taken between crossed prisms of a polarizing microscope, with the exception of "f." These photomicrographs are representatives from each group showing various degrees of degeneration. Rat 108 (fig. 1, a) shows the most degeneration, while rat 127 (fig. 1, h) shows the least. The latter, however, does show some degeneration of the myelin sheaths, notwithstanding the fact that the commercial fox chow was intended to be used in order to obtain normal rat tissues. When degeneration occurs, normal myelin apparently breaks down into isotropic compounds which appear black in the photomicrographs. Normal myelin, being anisotropic, appears white in the photomicrographs. In rat 111 (fig. 1, b), which had received a supplement of 5 μg. of thiamine for thirty-two days, there appears less degeneration in the myelin sheaths than in rat 108 (fig. 1, a). Rat 111 (fig. 1, b) corresponds closely to rat 114 (fig. 1, c). Rat 117 (fig. 1, d), receiving 5 μg. of thiamine and 1 mg. of thiamine analogue with a supplement of 5 additional μg. of thiamine the last sixteen days, shows less degeneration than rat 111 (fig. 1, b). Figures 1, e and 1, f of rat 120, receiving 1 mg. of thiamine analogue and 5 μg. of thiamine for the last twenty-nine days, are photomicrographs taken between crossed and uncrossed prisms, respectively. By the latter method, one cannot detect degeneration, while by the former, the amount of degeneration corresponds closely to that of rats 111 and 114 (fig. 1, b and 1, c). Rat 124 (fig. 1, g), receiving 50 μg. of thiamine, shows slightly more degeneration than rat 127 (fig.

TABLE 2—Results of the Marchi Method of Staining Tissues from Two Series of Rats Receiving the Thiamine-Free Basal Diet* with Daily Supplements of Thiamine and the *o*-Aminobenzyl Analogue** of Thiamine

Series 1	Series 2	Liver	Kidney	Heart	Sciatic nerve	Spinal cord	Brain
Group A							
108		0	0	Trace	+ +	+ + +	+
110		0	0	0	+	+ +	+
109		+	0	0	Trace	+	Trace
111		+	0	0	Trace	+	Trace
	145	Trace	0	Trace	+	+	0
	147	Trace	0	Trace	+	+	+
Group B							
112		+ +	0	0	+	+ +	+
114		+ + +	0	Trace	Trace	+ +	Trace
113		+	0	0	Trace	+	Trace
115		0	0	0	Trace	+	0
	149	Trace	Trace	Trace	+	+	+
	150	+	0	Trace	+	+	+
Group C							
116		+ +	0	0	Trace	+	0
118		+	Trace		+		Trace
117		+ +	0	0	Trace	+	Trace
119		+	0	0	Trace	0	0
	153	Trace	0	Trace	+	+ +	+
	154	Trace	Trace	Trace	+	+	+
Group D							
121		Trace	0	Trace	+	+ +	Trace
122		0	0	Trace	+	+ +	Trace
120		+	0	0	0	Trace	Trace
123		Trace	0	0	Trace	+	Trace
	160	0	0	Trace	+	+	+
Group E							
124		+ + +	0	0	+	+	0
125		+ + +	0	0	Trace	+	0
126		+ +	0	0	Trace	+	0
	162	+	+	Trace	+	+	Trace
	163	Trace	Trace	Trace	+	+	+
Group F							
127		0	0	0	Trace	Trace	0
128		0	0	0	+	+	0

*Group F received a commercial fox chow *ad lib.* **o-aminobenzyl-(3)-4-methyl-5-β-hydroxyethyl-thiazolium chloride hydrochloride.

+ + + = advanced fatty change, 50 per cent or more of area or number affected. + + = marked fatty change, 25 to 50 per cent of area or number affected. + = moderate fatty change, less than 25 per cent of area or number affected. Trace = slight fatty change, only enough for identification. 0 = no fatty change.

1, h), as evidenced by the swollen myelin sheaths.

In conjunction with the polarized light studies, small portions of liver, kidney, heart, sciatic nerve, skeletal muscle, spinal cord, and brain were placed in Muller's fluid. The Marchi method of staining[8] was followed, except that dioxane was used instead of alcohol for dehydration. The tissues were sectioned 20 μ in thickness.

Table 2 is a summary of the pathologic changes found in the tissues that were stained by the Marchi method. The degeneration of the myelin sheaths checks closely with the polarized light studies. Figure 2, which shows the degenerated myelin sheaths of the sciatic nerve as black, corresponds with figure 1, a (rat 108).

Myelin sheath degeneration in the spinal cords was general in all the rats. These findings were similar to those found in the corresponding sciatic nerves. As a rule, the lesions in the spinal cords appeared more marked than in the sciatic nerves. Figure 3 shows a cross section of the spinal cord of rat 108. A majority of the myelin sheaths appears to be degenerated.

Myelin sheath degeneration was seen in the brain medulla of all the rats except those in group F receiving the commercial fox chow and group E in series 1 receiving 50 μg. of thiamine daily. Figure 4 shows myelin sheath degeneration in the brain medulla of rat 112. Many of the sheaths appear unaffected while others have become deeply stained by the osmic acid.

Many of the livers showed a fatty change in the hepatic cells around the central veins, of which figure 5 is an example. This condition was present in all the livers (table 2) except from those rats receiving the commercial fox chow and those receiving thiamine-free diets, with the exception of rats 121, 145, and 147, which showed only a trace. The liver from rat 115, which had received 10 μg. of thiamine per day for the last twenty days, did not show a fatty change.

A small amount of fatty change was seen in the kidneys of rat 118 (series1) and of rats 149, 154, 162, and 163 (series 2). The Marchi staining method also revealed traces of a fatty change in the heart (table 2). All the hearts from series 2 were affected while the degenerative changes occurring in series 1 were inconsistent. Degenerative changes in the skeletal muscles were not detected by this staining method.

Portions of tissue corresponding to those taken for the Marchi staining method were sectioned and stained according to the hematoxylin and eosin method, except that a 1 per cent solution of triosin in 90 per cent alcohol was used instead of eosin. In thiamine deficiency, degenerative changes may occur in skeletal muscles.[7] Necrosed skeletal muscle fibers are often replaced by fibrous connective tissue,[8] which triosin differentiates by staining orange-red.

The tissues of the rats on the thiamine-free diet showed dehydration with atrophy. Figure 6 illustrates this condition as it occurred in the liver of rat 108. It is well to note the number of nuclei per unit of space. Similar changes were observed in the livers of all rats receiving the thiamine-free diet.

A fatty change, indicated by the appearance of large, clear vacuoles in the hepatic cells, was observed in rats receiving thiamine-deficient diets and also in those receiving 50 μg. of thiamine daily. These vacuoles corresponded in size and location to the large black droplets found in the tissue stained by the Marchi method (table 2). Figure 7 is a photomicrograph showing a fatty change in the hepatic cells around a central vein (rat 109). This rat was supplemented with 5 μg. of thiamine daily for thirty-two days, after receiving the thiamine-free diet for forty days.

Nerve cells in the gray matter of the brain medulla and spinal cord of all the rats, except those receiving the commercial fox chow, showed various degrees of degeneration. The number of degenerated cells was proportional to the amount of degeneration found in the sciatic nerves with the polarized light and Marchi methods. Figure 8 is a photomicrograph showing the degenerated nerve cells. These cells have lost all detail, with the exception of two normal cells toward the bottom of the picture.

Degenerative changes were observed in the skeletal muscles of the rats from all the groups in both series except the one receiving commercial fox chow. The skeletal muscles of the rats receiving the thiamine-free diet were atrophied. The rats receiving the 5 μg. supplements of thiamine in groups A and D showed less atrophy than those receiving the thiamine-free diet, an increase of connective tissue, and a loss of muscle striations. Less tissue atrophy was observed in the rats receiving more than 5 μg. of thiamine daily, with none appearing in the rats receiving 50 μg. daily.

The degenerative changes in the skeletal muscle fibers of rat 114 are illustrated in figure 9. The light areas in the photomicrograph show the early changes of degeneration in the muscle fibers. These areas are stained blue with hematoxylin and triosin in contrast to the normal pink fibers. Advanced degenerative changes of the skeletal muscle (rat 117) are seen in figure 10. Calcification, giant cells, and connective tissue proliferation are present. The rats of group E, receiving 50 μg. of thiamine daily, showed changes in the muscle fibers similar to figure 9.

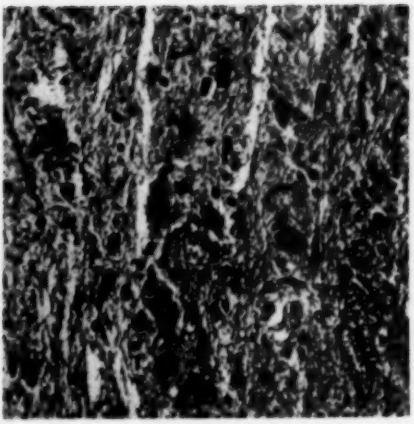

Fig. 8—Brain medulla of rat 108 showing degenerated nerve cells. Hematoxylin-triosin. x 300.

Fig. 9—Skeletal muscle of rat 114 showing an early degenerative change. Hematoxylin-triosin. x 300.

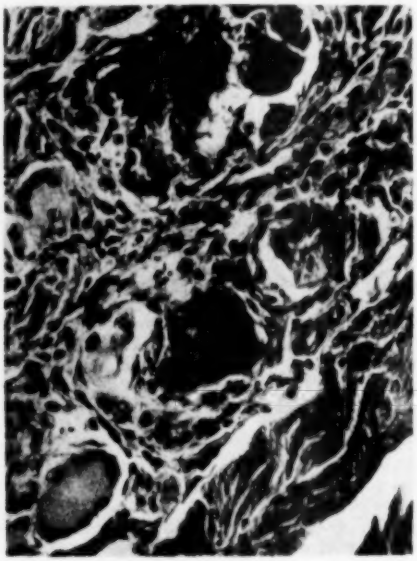

Fig. 10—Skeletal muscle of rat 117 showing calcification, giant cells, and connective tissue proliferation. Hematoxylin-triosin. x 300.

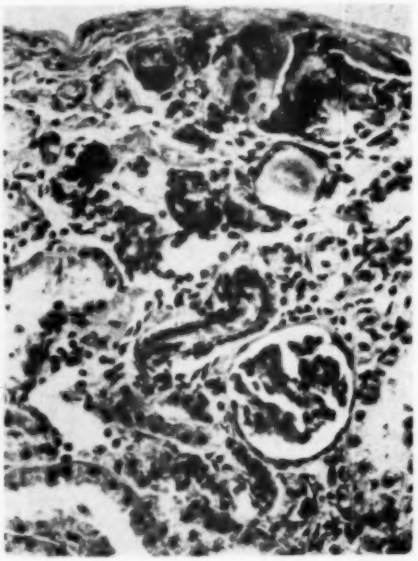

Fig. 11—Kidney (cortex) of rat 117 showing calcification. Hematoxylin-triosin. x 300.

The kidneys from the rats in groups A and D of both series were atrophied. Albuminous degeneration was present in the tubule cells throughout the kidneys of all the rats except those from group F, which received the commercial fox chow. Calcified areas in the cortex of the kidneys, surrounded by connective tissue proliferation, and hyaline casts in the tubules were found in rats 114 and 149 (group B), 117, 118, and 154 (group C), and 124 and 162 (group E). The calcified areas are close to the capsule. Figure 11 shows this condition in rat 117. The hyaline casts appear in the tubules, as shown in figure 12. The kidney of rat 118 showed the most severe degenerative changes of any rat which included large areas of necrosis in addition to calcification, connective tissue proliferation, hyaline casts in the tubules, and albuminous degeneration of the tubule cells. These alterations may account for the small weight gain of rat 118 as seen in table 1.

The hearts appeared normal with hematoxylin and triosin with the exception of a few myocardial hemorrhages.

DISCUSSION

The pathologic changes in the various rat tissues cannot be attributed to the *o*-aminobenzyl analogue of thiamine. By histologic comparison, the tissues from rats receiving the analogue cannot be distinguished from their controls. It is true, however, that considerable change had occurred in the tissues, but some factor or factors other than the analogue must be involved because the control animals were also affected.

The fatty change observed in the livers of rats receiving thiamine-deficient diets for approximately thirty-five days was absent in those receiving the thiamine-free diet over the same period of time. Although the daily administration of 50 μg. of thiamine is considered adequate for normal growth, the rats in group E, receiving this level of thiamine daily, also showed a fatty change in the liver around the central veins. Therefore, it appears that some factor or factors other than thiamine, either present or deficient in the diet, may have produced the fatty change in the liver.

Central necrosis of the liver lobules has been observed in cases of beriberi.[17] This condition was attributed to cardiac insufficiency which, in turn, impaired the venous circulation and later caused the central necrosis. Cardiac insufficiency may have caused the fatty change in the liver of the rats receiving thiamine-deficient diets. The demands upon the heart of the rats receiving the thiamine-free diet may have been lessened by the decreased food consumption and decreased activity to permit an apparent normal functioning of the organ.

The degenerative changes in the skeletal muscle, as loss of striations, hyaline formation, and necrosis with connective tissue proliferation and calcification, were observed in every animal except those receiving the thiamine-free diet and the commercial fox chow. These degenerative changes resemble those described in skeletal muscle under the name of Zenker's waxy degeneration,[18] which may occur in typhoid fever, tetanus, pneumonia, and other infections. Wells[19] states that Zenker's waxy degeneration in skeletal muscle is due to an increased accumulation of acid by a defective oxygen supply. This condition was produced in muscle fibers by the application of a weak solution of lactic acid and by the excessive stimulation of muscles. Under these conditions, the acid accumulated faster than it was removed. Since the lactic acid in the tissues increases with the increased pyruvic acid concentration in thiamine-deficient animals,[20, 21] it appears that degenerative changes in the skeletal muscle, as illustrated in figures 9 and 10, may have been caused by an increased acid concentration in the tissues. Runnells[19] states that calcification may accompany hyalinization where necrotic areas are being encapsulated (fig. 10).

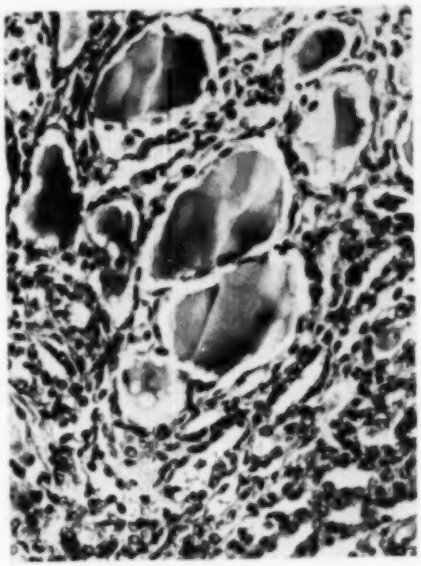

Fig. 12—Kidney (medulla) of rat 117 showing hyaline casts in tubules. Hematoxylin-triosin. x 300.

The myelin sheath degeneration which was observed in the rats receiving an adequate amount of thiamine (groups E and F) suggests that a lack of thiamine may not be the cause of myelin sheath degeneration. Engel and Phillips[11] found that myelin sheath degeneration was seldom observed in a rat on a thiamine-deficient, but otherwise adequate, diet. The same investigators[10] demonstrated that riboflavin was necessary in the chick diet to prevent myelin from degenerating. Publications by Sutton and coworkers[2] and Setterfield and Sutton[7] suggest vitamin A deficiency as the causative factor in producing myelin sheath degeneration. Eveleth and Biester[12] concluded that neither vitamin A nor the B-complex was responsible for the myelin sheath degeneration in the spinal cords and peripheral nerves of swine.

The number of factors stated in the literature review as causing myelin sheath degeneration leads one to believe that the diets used in these experiments may not have been as complete as originally believed when the experiments were run. It is true that satisfactory growth responses have been obtained in animals receiving purified diets, but growth cannot be considered as the only criterion in evaluating the completeness of such diets. Histopathologic changes must be considered as they have been shown to vary with the diet. Some diets are designed for short term experiments, and pathologic changes are, therefore, more likely to occur if the experiment is prolonged. With the recent advances in nutrition and the discovery of new nutritional factors, it appears that some of the histopathologic changes occurring in the various experimental animals may be corrected simply by feeding more complete diets.

SUMMARY AND CONCLUSIONS

1) The *o*-aminobenzyl analogue of thiamine, *o*-aminobenzyl-(3)-4-methyl-5-β-hydroxyethylthiazolium chloride hydrochloride, did not produce additional histopathologic changes when 1 mg. was given daily to rats receiving the thiamine-free diet and to rats receiving 5 μg. of thiamine daily in addition to the thiamine-free basal diet.

2) The etiology of myelin sheath degeneration is not limited to thiamine deficiency, as the myelin sheaths from rats receiving an adequate amount of thiamine showed degeneration.

3) The findings observed by the Marchi method of staining checked closely with the polarized light and with the hematoxylin-triosin staining method.

4) Animals receiving a purified basal diet may show histopathologic changes even when growth has occurred at an apparent normal rate.

References

[1]Swenson, M. J.: The Effect of a Thiamin Analogue on Growth and on the Thiamin Content of Rat Tissues. Am. J. Vet. Res., *10*, (1949): 77-84.

[2]Sutton, T. S., Setterfield, H. E., and Krauss, W. E.: Nerve Degeneration Associated with Avitaminosis A in the White Rat. Ohio Agric. Exper. Sta., Bull. 545, 1934.

[3]Baird, Thelma T.: Polarized Light in Photomicrography, Its Principles and Applications. J. Biol. Photographic A., *5*, (December, 1936) :43-59.

[4]Lee, Jack, and Sure, Barnett: Avitaminosis. XIX. Nerve Degeneration in Albino Rats as Studied by the Freezing-Drying Method and Polarized Light with Deficiency of Vitamin A or of Vitamin B. Arch. Path., *24*, (1937): 430-442.

[5]Prickett, C. O., Salmon, W. D., and Schrader, G. A.: Histopathology of the Peripheral Nerves in Acute and Chronic Vitamin B_1 Deficiency in the Rat. Am. J. Path., *15*, (1939): 251-259.

[6]Hassin, George B.: Histopathology of the Peripheral and Central Nervous Systems. Paul B. Hoeber, New York, 2nd ed., (1940): 27-31.

[7]Setterfield, H. E., and Sutton, T. S.: The Use of Polarized Light in the Study of Myelin Degeneration. II. The Degeneration of Myelinated Nerves in Avitaminosis A in the White Rat. J. Nutr., *9*, (1935) :645-655.

[8]Davison, Charles, and Stone, Leo.: Lesions of the Nervous System of the Rat in Vitamin B Deficiency. Arch. Path., *23*, (1937): 207-223.

[9]Shaw, James H., and Phillips, Paul H.: Neuropathologic Studies of Acute and Chronic Thiamine Deficiencies and of Inanition. J. Nutr., *29*, (1945): 113-124.

[10]Phillips, Paul H., and Engel, Ruben W.: The Histopathology of Neuromalacia and "Curled Toe" Paralysis in the Chick Fed Low Riboflavin Diets. J. Nutr., *16*, (1938): 451-463.

[11]Engel, Ruben W., and Phillips, Paul H.: A Lack of Nerve Degeneration in Uncomplicated Vitamin B_1 Deficiency in the Chick and the Rat. J. Nutr., *16*, (1938): 585-596.

[12]Eveleth, D. F., and Biester, H. E.: The Significance of Myelin Sheath Degeneration and Its Relation to Incoördination. Am. J. Path., *13*, (1937): 257-266.

[13]Evans, C. A., Carlson, W. E., and Green, R. G.: The Pathology of Chastek Paralysis in Foxes. Am. J. Path., *18*, (1942): 79-90.

[14]Green, R. G., Carlson, W. E., and Evans, C. A.: A Deficiency Disease of Foxes Produced by Feeding Fish. J. Nutr., *21*, (1941): 243-256.

[15]Vedder, E. B.: The Pathology of Beriberi. In "The Vitamins" by Williams, R.R., A.M.A., Chicago, Ill., (1939): 179-191.

[16]Mallory, F. B.: Pathological Technique. W. B. Saunders Co., Philadelphia, Pa., (1938): 237-239.

[17]Eddy, Walter H., and Dalldorf, Gilbert: The Avitaminosis. Williams and Wilkins Co., Baltimore, Md., 3rd ed., (1944): 186.

[18]Boyd, William: Textbook of Pathology. Lea and Febiger, Philadelphia, Pa., 5th ed., (1947) : 133.

[19]Wells, H. Gideon: Chemical Pathology. W. B. Saunders Co., Philadelphia, Pa., 5th ed., (1925): 439-440.

[20]Fulton, John F.: Howell's Textbook of Physiology. W. B. Saunders Co., Philadelphia, Pa., 15th ed., (1947): 1119.

[21]Harrow, Benjamin: Textbook of Biochemistry. W. B. Sanders Co., Philadelphia, Pa., 4th ed., (1946): 149.

[22]Runnells, Russell A.: Animal Pathology. Iowa State College Press, Ames, Iowa., 4th ed., (1946): 97.

Histomoniasis (Enterohepatitis) in Turkeys
I. A Procedure for the Screening and Testing of Drugs

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IN SEARCHING for effective chemotherapeutic agents for the control of histomoniasis (enterohepatitis) in turkeys, one is immediately presented with the problem of finding a method of reproducing the disease which is satisfactory for screening and testing drugs. Choice of a particular method depends, to a great extent, on several factors. First, it depends on how rapidly one wishes to screen drugs; second, it depends on the facilities and the availability of poults. Time is also an important factor in screening drugs, due to the seasonal occurrence of histomoniasis and also the seasonal availability of poults.

Methods available at one's disposal are: first, exposure of the birds to an infected range. This is cumbersome, slow, and very difficult to control. Another method consists of feeding infected Heterakis ova, a technique which is desirable from the standpoint of reproducing the disease in its natural form. However, it may not always produce a high mortality and is more apt to have a variable period of incubation. These factors may, in some instances, be desirable, depending entirely on circumstances. The most serious handicap to this method is the difficulty in securing sufficient numbers of the infected ova for large scale screening trials.

A very desirable method consists of artificial cultivation of histomonads, as practiced by DeVolt,[1] with subsequent inoculation of turkeys. The merits of this method are many, viz., no interfering organisms, controlled amount of inoculum, and reproducible results. The only disadvantage is the difficulty of culturing the organisms in sufficient numbers for large scale experiments and, possibly, maintaining the virulence of a culture.

The rectal injection method developed for these studies was used because of experience with the technique, the relatively high percentage of mortality obtained, the speed with which the work could be conducted, and the similarity of the experimentally produced disease with the naturally occurring condition. Another marked advantage is the ready availability of the inoculum once the infection is started. An objection against this method, however, is the difficulty in controlling the dosage, a fact which may cause a drug to be undervalued in some instances, due to an excessively dense population of organisms in the inoculum. Another disadvantage is that the culture must be maintained by serial passage through poults.

The basic idea of the rectal injection method is not new, since it has been used by Tyzzer and Fabyan,[2] Tyzzer and Collier,[3] Delaplane,[4] and others. Delaplane was able to obtain a mortality of 84 per cent, but none of the investigators attempted large scale experiments.

METHOD

The technique of preparing the inoculum for

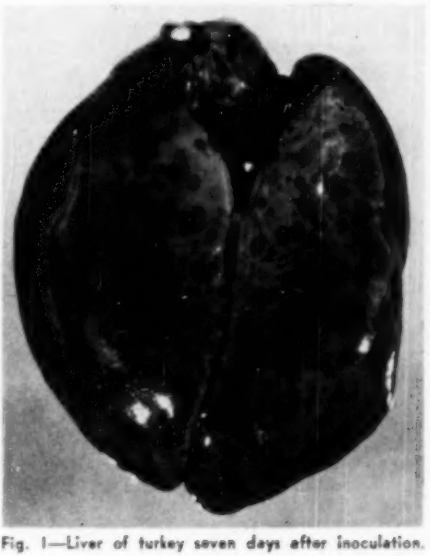

Fig. 1—Liver of turkey seven days after inoculation.

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The investigation herein reported was, in part, made possible through support granted by Merck and Co., Inc., Rahway, N. J., who also furnished the majority of the drugs.

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the rectal injection consisted of obtaining livers from poults recently dead or destroyed at the height of the disease. These livers were immediately thinly sliced with a knife and then pressed through a fine screen (16 meshes to the inch). The resulting material was then

Fig. 2—Liver of turkey twelve days after inoculation.

diluted with saline or beef extract broth, to a consistency thin enough for injection with a syringe.

The syringe used for injection was an ordinary 50-cc. size, with a rubber tube attached. The tube was cut several inches from the syringe and a 5-cc. volumetric bulb interposed. This arrangement allowed measurement of the inoculum, without drawing any into the barrel of the syringe and thus causing the plunger to stick. The length and diameter of the tube could be varied at will, depending on the size of the turkey to be injected. The liver inoculum was injected immediately after preparation.

The injections were made by 1 person suspending the bird by the legs and another operator inserting the rubber hose into the rectum to the ileocecal junction, where 5 cc. of the material was slowly discharged. At this point, care must be exercised to avoid rupturing the gut, with a fatal peritonitis resulting. Five cubic centimeters of the liver material was injected into each bird regardless of size.

The plan for the use of this method was to infect the birds and then to administer the drug at varying periods of time and for varying lengths of time. The poults were weighed at the beginning and at the end of an experiment.

When critical experiments were carried out, feed consumption was measured, birds weighed every other day, and symptoms, such as yellow droppings, were noted. To gain further information on a critical study, four groups of birds were used as follows: treated infected, treated uninfected, untreated infected controls, and untreated uninfected controls.

In the preliminary trials on a drug, only an untreated infected control group was used, since drug activity could be just as accurately detected, and additional groups were added provided the drug warranted such a procedure. This procedure was adopted as a measure of economy.

All dead birds were autopsied and carefully inspected for lesions. Serum-dextrose agar slants were inoculated routinely from the liver as a precaution against concurrent infection. Occasionally, the heart and spleen were likewise cultured. Also, routine wet smears were made from the cecums for microscopic identification of organisms present. Cecal lesions were also noted and recorded.

Since the immunity obtained by a turkey

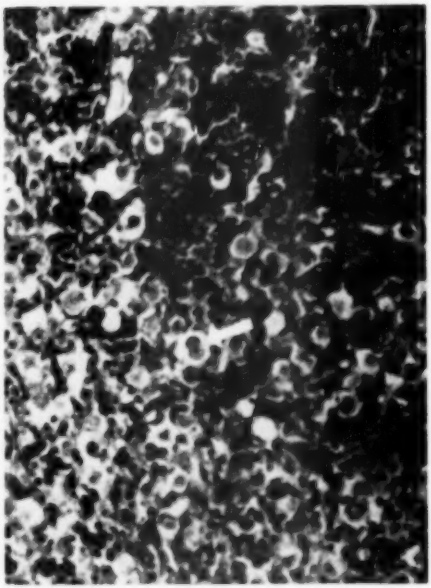

Fig. 3—Liver seven days after inoculation. Note histomonad dividing in right center portion of picture (arrow). × 260.

from infection with histomoniasis is not clearly understood, some of the experiments were designed to furnish information along these lines. In some of the drug trials, one half of the infected, treated survivors were destroyed, autopsied, and examined for evidence of healing lesions in the liver and cecums. Sections of the liver were taken for histologic examination. The other half of the surviving turkeys were given a challenging inoculation in an attempt to demonstrate development of immunity. The birds were divided carefully on the basis of weight changes and symptoms so as to get a more accurate picture of the autopsied turkeys and, likewise, of the challenged birds. The details and results of the immunity studies will be reported in Part II. Some of the more interesting findings covering the pathologic changes will be discussed later in this paper.

Mention was made previously that the density of the inoculum may vary. It was observed that when a culture was serially passed through turkeys, the incubation period became much shorter and deaths occurred earlier; also mortalities were much higher. It followed that, in general, after a culture had been serially passed three times, maximum virulence was reached (*see* table 1).

In table 1, it will be noted that the number of serial passages varied from one to two times. Accordingly, deaths did not occur as early when the culture was passed three or more times. Experiment 6 in this table shows a mortality of 90 per cent, with deaths occurring as early as the sixth day. This is understandable because the culture was obtained from a field outbreak in which a very high mortality occurred.

It is felt the term "virulence" may not be correctly applied to this phenomenon. These following remarks should be prefaced with the statement that it is well known that a culture of organisms may become more pathogenic upon serial passage. In addition to the apparent increased pathogenicity with subsequent passages, another factor may be in part responsible for the higher mortalities and shorter incubation period. This factor is the belief that with serial passage the young, rapidly growing histomonads become concentrated in the liver and subsequently in the inoculum.

To substantiate this belief, it was noted that turkeys dying from field outbreaks contained old, healing (*see* fig. 2), as well as

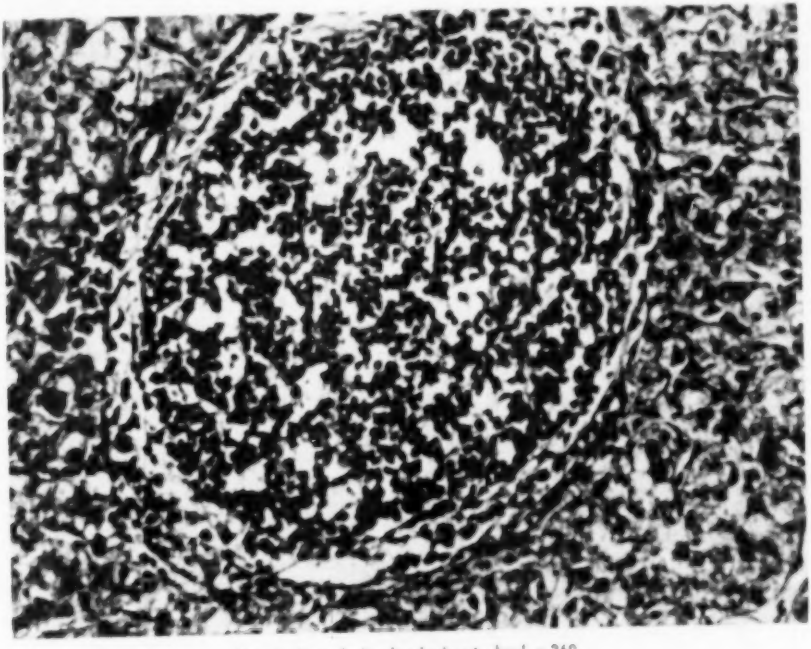

Fig. 4—Liver lesion beginning to heal. x 260.

fresh, hemorrhagic lesions in the liver. It would be logical to assume that 5 cc. of inoculum, made from livers with many healing lesions, would contain fewer organisms than a liver composed of recent active lesions, such as shown in figure 1 and microscopically in figure 3.

Figure 3 is a photomicrograph of the liver of a turkey which died on the seventh day after inoculation. The picture is representative of practically the entire liver. The liver cords are broken and disrupted with numerous histomonads dispersed throughout. The organisms are distinguished by a dark nucleus surrounded by a light halo. A histomonad can be seen dividing in the middle right portion of the photograph (*see* arrow). The small dark cells in the upper right hand corner are erythrocytes. Microscopic examination of liver tissues from birds dying in twelve days or longer shows more necrotic tissue located in foci, and the organisms tend to take a more acid stain, which agree with Tyzzer's[5] findings. Younger organisms appear to take the basic stains more readily. These findings tend to support the belief that recent, acute lesions contain large numbers of young, rapidly multiplying organisms and may be a factor in causing high mortalities as well as increased pathogenicity.

A practical analogy of this situation was observed when the livers of relatively avirulent field cases were brought to the laboratory and serially passaged. On the first passage, the poults usually died about the fifteenth day. The second passage killed the poults in about twelve days, and the third passage in eight days. In contrast, a poult, secured from a flock suffering heavy losses in young birds, may produce a culture capable of killing in eight days on the first passage. A glance at table 1 shows how serial passage tends to increase the concentration of the organisms in the liver or to increase the pathogenicity of a given volume of macerated liver used for inoculation. There is a possibility, then, based on the above observations, that the higher mortalities and shorter incubation periods may have been due to increased numbers of organisms per unit as well as the increased virulence with serial passage.

It was noted that poults surviving an experiment recovered rapidly, even though they had shown rapid weight loss and yellow droppings. Some of these birds were autopsied eighteen to twenty-three days after inoculation and the livers examined both grossly and microscopically. At this time, the lesions were healing rapidly, many of the smaller ones only visible as a dimple on the surface. Microscopically, those lesions appeared as foci of lymphocytes (*see* fig. 4) surrounded by regenerating liver cells. Besides the lymphocytes, the lesions contained a few liver cells, macrophages, and cellular débris.

Figure 5 shows the same type lesions, which have progressed further in the healing process. The healed area is composed of a few lymphocytes and young liver cells. The light area in the regenerated area is an artifact. Note the faint line running di-

TABLE 1—Summary of Mortality of Infected Untreated Controls

Exp. No.	No. Serial Passages	No. Per Exp.	Av. Wts.	Days After Inoculation													% Mortality
				5	6	7	8	9	10	11	12	13	14	15	16	17	
1	1	20	1540								1	3	1	3	3	2	65
2	1	20	1835							1	1	2	5	3	3	1	80
3	2	11	1650						2	1	1	1	1	1			63
4	1	20	1644				1	3		1	5	2		1			65
5	1	20	1640				2	1	3	6	1	2		2			85
6	2	20	1790		3	2	2	3	7	1							90
7	1	20	980				2		2	3	3	1			1	1	65
8	1	20	1330				1	2		6	2	2	2	1	1	1	90
9	3	20	1007		4	5	3	2		1	1	1					85
10	4	23	650		3	10	8	1	1								100
11	5	10	884		3	4	2	1									100
12	6	20	1685		2	7	7	3					1				100
13	7	19	1829			3	5	5	3	2							95
14	8	20	1896			5	10	4	1								100
15	10	20	1681		1	4	8	3	3	1							100
16	2	10	1000							1	3	2	2	1	1		100
17	9	10	1590	1	1	3	2	2	1								100
18	5	10	1650			3	4	2	1								100
19	6	7	1790		2	1	2	2									100
20	7	7	1530	1	2	3	1										100

agonally through the picture, which demarcates the regenerated liver tissue from the normal. As the process of regeneration proceeds, liver cells progressively replace the area destroyed by histomonads until it resembles that shown in figure 5.

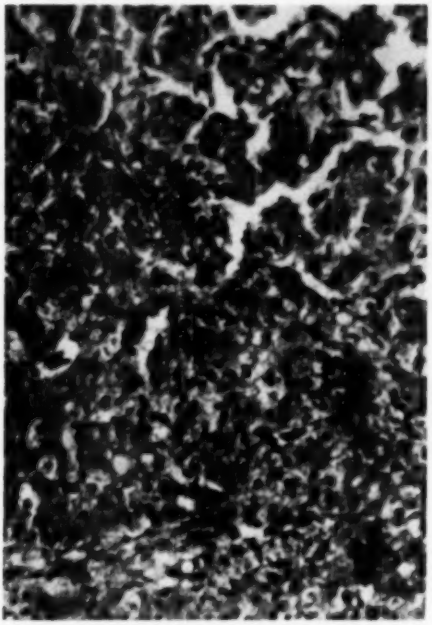

Fig. 5—Liver lesion practically healed. Note faint line diagonally across picture indicating margin of lesion. x 260.

The entire process of liver destruction, including invasion, necrosis, healing, and regeneration appears to proceed at a very rapid rate in experimentally produced histomoniasis.

SUMMARY

1) A method for producing histomoniasis is presented, which involves the rectal injection of minced liver tissue from infected poults.

2) The procedure provides a rapid, satisfactory means for screening and testing drugs against histomoniasis.

3) The higher mortality and shorter incubation period, resulting from serial passage of the culture, may be due to greater concentration of the histomonads, as well as increased virulence.

4) Liver lesions of turkeys recovering from the experimental disease regenerate very rapidly and without fibrosis.

References

[1]DeVolt, H. M., and Holst, A. P.: Preliminary Report of the Preventive Action of Vioform Against Enterohepatitis (Blackhead) of Turkeys. Poult. Sci., 27, (1948):356-358.

[2]Tyzzer, E. E., and Fabyan, M.: Further Studies on "Blackhead" in Turkeys with Special Reference to Transmission by Inoculation. J. Inf. Dis., 27, (1920):207-239.

[3]Tyzzer, E. E., and Collier, Jane: Induced and Natural Transmission of Blackhead in Absence of Heterakis. J. Inf. Dis., 37, (1925):265-276.

[4]Delaplane, J. P.: Etiological Studies of Blackhead (Enterohepatitis) in Turkeys. Rhode Island Exper. Sta., Bull. 233, Jan., 1932.

[5]Tyzzer, E. E.: Developmental Phases of the Protozoan of "Blackhead" in Turkeys. J. Med. Res., 40, (1919):1-30.

Histomoniasis (Enterohepatitis) in Turkeys
II. Chemotherapy of Experimental Histomoniasis

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THIS PAPER is a brief presentation of the observations and results obtained in the experimental chemotherapy of histomoniasis of turkeys.

The chemotherapy of protozoan diseases has involved many classes and types of compounds. From this wide variety of agents, only a relatively few groups of compounds are practical in treating the various maladies caused by protozoan infections. The turkey industry is still without the benefit of any good therapeutic agent to combat histomoniasis.

Some of the drugs used in this study were chosen because of their effectiveness in treating certain conditions in man and animals, e.g., arsenicals in trypanosomiasis. Others were chosen because of previous reports of their use in histomoniasis. Possibly, the only exception was sodium fluoride which was given a trial more or less as a matter of chance.

Space does not permit a detailed discussion of the projected mechanism of action of the drugs affecting protozoan organisms, or of the handicaps which face advances in protozoan chemotherapy. One might mention specifically, concerning the therapy of histomoniasis, the lack of knowledge involving the physiology of *Histomonas meleagridis* and concerning host-parasite relationship. At present, the necessity of finding better agents to treat diseases increases the proclivity to continue the use of trial and error methods. As a result, most present-day investigations consist of screening procedures.

METHOD USED IN DETERMINING DOSE OF DRUG

The selection of a dosing schedule for the drugs was a matter of considerable importance. Very little was known about the tolerance in turkeys to the chemotherapeutic agents used in these studies.

To avoid difficulty in interpreting results, it was thought best to arrive first at what was thought to be a nearly maximum tolerated dose, which would be used in therapy trials, rather than to give varying doses. The thought was to administer a dose just below the toxic level as determined in normal poults. It was hoped this procedure would, in most instances, give the drug the best opportunity to show protozoacidal activity. If the drug showed activity, the dose was to be reduced and tried again.

To arrive at what was thought to be a maximum tolerated level, a wide range of doses was given to poults (table 1a). The procedure was repeated, using levels above and below the apparent minimum level shown in the first trial. In the second trial, more poults were used in each group for greater accuracy.

Regardless of whether the drugs were to be administered orally or parenterally, all were run through this procedure with the exception of the sulfonamides, iodine vermicide, and vioform. All birds dying from the effect of the drug were autopsied, and gross and microscopic changes in the tissues were recorded.

In the therapy trials, all poults were infected by use of the rectal injection method as described previously.[1]

EXPERIMENTS, OBSERVATIONS, AND RESULTS

In carrying out the following experiments, interest was centered primarily around whether or not a drug was effective in the treatment of histomoniasis. To measure this activity, mortality was considered the best criterion. Since the chemotherapy of histomoniasis is principally an "all or none" proposition, it was felt other criteria furnished information of lesser importance. In the early part of the therapy trials, not all birds were weighed at the termination of an experiment. This procedure was followed because most of the experiments were designed merely as screening procedures and were not meant to be conclusive in any manner.

In the first experiment, tryparsamide was used intravenously at 1.25 Gm. per kilogram. The drug was administered to five lots of 10 birds each as follows: on the day of infection; and on the second, fourth, sixth, and eighth days, respectively. Each lot of birds was further divided, one lot receiving a single dose, the other receiving a dose every other day for

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a total of three doses. By arranging the dosing schedule in this manner, efficacy of repeated dosing and early therapy could be evaluated. The experiment was repeated, except that 12 birds per lot were used instead of 10 and a total of four lots instead of five. The results of these two experiments are summarized in table 1.

The mortality was lower in the groups receiving the drug the first few days after infection. These results were expected, since the experience of others, in treating this disease, has shown that late therapy appears to be of little value. The mortality in the untreated infected control groups was 65 and 80 per cent, respectively, with an average of 75 per cent. The higher mortality in controls in the second experiment was thought to be due to increased virulence of the inoculum.

The results of the two experiments seem to indicate that late therapy is of little value and also that multiple doses are more effective than single. This is graphically demonstrated in figures 1 and 2. Further, the results indicate that if the drug is given within the first three days after infection, some therapeutic activity is shown. This will be clearly demonstrated in experiments to be described later.

The drugs summarized in table 3 were used in the same manner as those in table 1. Since little or no therapeutic effect was demonstrated, further trials were thought unnecessary. The immune blood, noted in table 3, was obtained from turkeys which had recovered from histomoniasis and then withstood six challenging injections of infective liver, given about three weeks apart. Ten cubic centimeters of the whole blood were given on the day of infection and on the following day. The mortality was 100 per cent, which indicates, rather clearly, that humoral antibodies in recovered turkeys were not present in high concentration or were not effective in preventing the disease.

TABLE 1—Summary of Experiments 1 and 2 Using Tryparsamide 1.25 Grams per Kilogram

Day of initial treatment following infection	No. doses of drug	Day and No. deaths after infection											Mortality	
		8	9	10	11	12	13	14	15	16	17	18	(No.)	(%)
0	1		1						1				2/11	18
0	3	1											1/11	9
2	1		1					1		1	2		4/11	36
2	3	1									1		3/11	27
4	1				1			1	2	2	1	1	9/11	82
4	3									1	1	1	3/11	27
6	1					2				2			9/11	82
6	3						1	1	2	3	1		6/11	55
8	1					1		1					2/5	40
8	3							1				1	2/5	40
Infected untreated controls	—				1	2	5	6	6	6	3	1	30/40	75

The drugs (table 4) were all given orally. Some had been tried previously using other routes of administration.

The oral therapy procedures will be discussed according to experiments, since, in most of the trials, drugs whose activity was known were used. Comparisons may be made more accurately since the mortality among the controls varied from one experiment to another; also, such factors as weight and environmental conditions may have altered results somewhat. Emphasis in the discussion will be placed on such drugs as stovarsol (acetarsone) and tryparsamide because of their therapeutic activity, and also because of their use in most of the trials.

TABLE 1 a—Preliminary Tryparsamide Toxicity Trial

No. birds	Ave. wt. (Gm.)	Dose in Gm./kg. (i. v.)	No. doses at three-day intervals	Toxic symptoms	Mortality	Ave. wt. gain in nine days (Gm.)
2	1050	0.5	3	None	0/2	416
2	998	1.0	3	None	0/2	185
2	1025	2.0	3	1 death	1/2	357
2	950	4.0	3	2 deaths	2/2	...
5	Controls					312

Sulfaquinoxaline, sulfamethazine, and iodine vermicide (*see* table 4, experiment 3) were not impressive in preventing deaths. Contrary to the usual findings, the turkeys receiving the sulfonamides early suffered a greater mortality than did those medicated on the second day after infection. However, this is not surprising, since deaths did not occur until the tenth day after infection either in treated or untreated groups.

The turkeys receiving iodine vermicide were given ½ oz. orally every other day for a total of eight doses. In addition, the birds were given 1 pt. of iodinated milk (1 oz. of diluted iodine per pint) daily in the drinking water. This amount furnished 70 mg. of iodine daily in addition to the 250 mg. given orally every other day. At the termination of the experiment, the survivors were in poor physical condition, shaggy in appearance, and with poor appetites. Contrary to the usual occurrence after infection, the turkeys were slow in regaining the appearance of normal birds.

The results in experiment 4 indicate sulfaquinoxaline may have a slight toxic effect based on comparison with the mortality in the control group. However, these results may be due to chance and could only be evaluated with repeated trials, involving larger numbers of birds. Compared with sulfaquinoxaline, stovarsol afforded 60 per cent greater protection, but the rather low mortality in the control group on a single trial makes such a conclusion hazardous.

The importance of repeating identical experiments can not be overemphasized, if one is to obtain reliable information. This is clearly shown when the results of the stovarsol-treated birds of experiments 4 and 5 are compared. There were 10 poults in each group. The same drug level in the feed and the same schedules were employed, yet the mortalities were 30 per cent and zero, respectively.

TABLE 2—Final Trypansamide Toxicity Trial

No. birds	Ave. wt. (Gm.)	Dose in Gm./kg. (i. v.)	No. doses at three-day intervals	Toxic symptoms	Mortality	Ave. wt. gain in nine days (Gm.)
5	1204	1.0	2	None	0/5	194
5	1428	1.75	3	None	0/5	255
5	1671	2.5	3	Depressed off feed	5/5	...
5	1610	3.0	2	All died 2nd inj.	5/5	...
5	Controls					262

TABLE 3—Summary of Results of Drugs Which Showed Little or No Therapeutic Value

Drug	Dose	Mortality	Control mortality
Neoarsphenamine	50 mg./kg. I.v.	23/24	17/20
Anthiomaline	4 mg./kg. I.p.	29/36	13/20
Eagle's compound	5 mg./kg. I.v.	15/30	17/20
Dimidium bromide	12 mg./kg. Subcut.	25/40	17/20
Pentamidine	10 mg./kg. Subcut.	25/30	18/20
Iodine vermicide	1 oz. orally	16/18	10/10
Immune blood	10 c.c. I.v. and Subcut.	10/10	10/10

In experiment 6, the results obtained were more in accord with later trials. The groups treated with stovarsol and trypansamide resulted in mortalities of 30 and 10 per cent, respectively. Autopsy of the dead birds revealed grossly normal cecums, indicating the drug was effective in preventing infection of the intestinal tract. The cecums of the infected untreated controls were all grossly affected. Likewise, the cecums of the sulfaguanidine- and streptomycin-treated birds were similarly involved. The survivors were weighed twenty-three days after infection, all showing, in general, normal weight gains.

The results in experiment 10 are different from any observed in the earlier trials in that all groups suffered high mortality, but the cecums of the treated birds were grossly negative, 67 and 30 per cent, respectively, as compared with 100 per cent affected in the control group. Further indication of drug activity is shown by deaths resulting two days earlier in the untreated birds.

These high mortalities may be the result of two factors: (a) The birds were lighter in weight than in most previous experiments, and (b) the culture was passed the fourth time. It was reported in a previous paper[1] that, in general, a culture after three serial passages reaches its maximum viru-

lence. In addition, young turkeys are known to be more susceptible to histomoniasis.

To repeat in part experiment 10, experiment 11 was started using the same concentration of stovarsol (0.75%), with medication begun two days before infection and

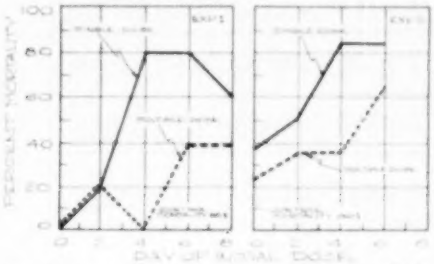

Fig. 1 and 2—Comparison of single versus multiple infections and early versus late therapy using tryparsamide (1.25 Gm./kg.).

maintained two days longer than in the previous trial. In addition, the following vitamins were added to the feed: nicotinic acid, pyridoxine, biotin, and folic acid. The amounts used were ten times the quantity prescribed for chicks by the subcommittee on poultry nutrition. In the experiment, the actual amounts were given per pound of feed: nicotinic acid, 80 mg.; pyridoxine, 16 mg.; biotin, 0.45 mg.; and folic acid, 9 mg.

The vitamins and stovarsol, each alone and together, were given to separate groups of birds. The mortality in the birds on stovarsol and stovarsol plus vitamins was 11 and 10 per cent, respectively, as compared to 100 per cent deaths in the control group. The mortality in the vitamin-treated group was 67 per cent, which indicates a possible benefit. Also included in this experiment was a group on 1 per cent vioform. The 100 per cent livability obtained on this drug in this trial shows very clearly the necessity for repeating experiments, as will be shown in experiments 12 and 14.

The results in experiment 12 were more conclusive in that 20 turkeys were used per group. Normal drug and normal control groups were also included. The weight gains in all control groups were not significantly different, and all birds were in good physical condition at the end of twenty-three days. Feed and water consumption were measured both in the infected and uninfected groups. All turkeys were weighed every other day and checked for symptoms of histomoniasis.

The results of these measurements indicated that birds ill with the disease, when on a drug such as stovarsol, lost weight and dropped off on feed consumption for several days, then recovered, and gained weight rapidly. The period of weight loss was not identical for all birds and, in general, was from the seventh to the ninth day after infection when the culture used for inoculation had been passed at least three times. Yellow droppings were most prevalent during this period. When the average weight of infected treated birds was plotted (*see* fig. 3), the curve was either less steep for a few days or, in general, did not show the gradual increase of the normal or uninfected groups. (The curves shown in fig. 3 were not taken from experiment 12 but, in general, are representative.)

Comparison of a normal growth curve with that representing infected birds does not show a true representation of both groups. The curve representing the infected control group levels off and drops rapidly as the birds lose weight and die, while the curve of the infected treated group indicates that some, or perhaps all, of the birds failed to gain weight somewhere during the period of a week, depending on the effectiveness of the drug. Actually, a part of the birds made normal weight gains while others either died or were very ill. The masking of the sick birds by the protected birds can not be shown advantageously in a graph such as figure 3.

Another cause of the difficulty in presenting the true picture is that the poults did not all become ill and lose weight on the

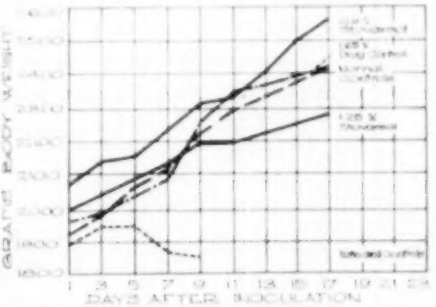

Fig. 3—Experiment 14. Weight comparisons during infection and treatment with stovarsol.

same day. Since the period of weight loss was usually two to four days, some of the birds were past this period before others started. The infected control curve in figure 3 is more representative of what may happen to the affected birds in a group on treatment. An analogy is seen in the birds

TABLE 4—Summary of Drugs Used Orally

Exp. No.	Drug Used	% Drug in Feed or Water	No. Birds	Av. Initial Wt. in g.	Drug Schedule (←→) Day & No. Deaths after Inoculation -2 -1 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15	% Mortality	% Showing Caecal Lesions at Autopsy: Right Caeca	Left Caeca	Mes.	Av. Indiv. Gain of Survivors
3	Iodine Vermicide	See Text	7	1856	←→ ←→ ←→ ←→ ←→ ←→ 1 ←→ 1 ←→	28	—	—	—	—
	Sulfamethazine	0.40	7	1502	←→ ←→ 1 1 1 1	57	—	—	—	—
	Sulfamethazine	0.40	7	1678	←→ 1 1	28	—	—	—	—
	Sulfaquinoxaline	0.10	9	1672	←→ ←→ 1 1 1	33	—	—	—	—
	Sulfaquinoxaline	0.10	7	1604	←→ ←→ 1	14	—	—	—	—
	Infected Controls	None	11	1650	2 1 1 1 1 1	63	—	—	—	—
4	Stovarsol	0.50	10	1490	←→ 1 ←→ 1 1	30	—	—	—	974
	Sulfaquinoxaline	0.10	10	1415	2 1 3 1 1 1	90	—	—	—	—
	Infected Controls	None	20	1644	1 3 1 5 2 1	65	—	—	—	—
5	Stovarsol	0.50	10	1728	←→ ←→	0	—	—	—	—
	Sodium Fluoride	0.75	10	1701	←→ 2 3 2 1 1 1	100	—	—	—	—
	Infected Controls	None	20	1640	2 3 7 1 4	85	—	—	—	—
6	Stovarsol	0.75	10	1936	←→ ←1— 1— 1→	30	0	0	100	784
	Sulfaguanidine	1.00	10	1627	←→ 2 4 1 2	90	70	100	0	0
	Streptomycin	40,000U/Day	10	1740	←→ ←1→	10	0	0	100	820
	Trypars amide	0.50	20	1790	3 2 2 3 7 1	90	80	100	0	1000
	Infected Controls	None								
9	Pentamidine	0.25g/gal.	10	1155	←→ 1 4 3 1 1	100	100	100	0	—
	Dimidium Bromide	0.25g/gal.	11	1225	←→ 4 5 2	100	100	100	0	—
	Eagle's Cpd.	0.175g/gal.	10	1070	←→ 1 5 2 2	100	100	100	0	—
	Infected Controls	None	20	1007	4 5 3 2 1 1 1	85	100	100	0	—
	Pent. Controls	0.25g/gal.	5	700	←→	—	—	—	—	155
	D. Bromide Contr.	0.25g/gal.	5	645	←→	—	—	—	—	239
	Eagle's Cpd. Contrs	0.175g/gal.	5	627	←→	—	—	—	—	238
	Normal Controls	None	5	718		—	—	—	—	119
10	Stovarsol	0.75	10	722	←→ 2 5 1 1	90	0	33	67	—
	Trypars amide	0.75	10	711	←→ 4 3 1 2	100	0	70	30	—
	Infected Controls	None	23	650	3 10 8 1 1	100	100	100	0	—

TABLE 4 (continued)—Summary of Drugs Used Orally

Exp. No.	Drug Used	% Drug in Feed	No. Birds	Av. Initial Wt. in g.	Drug Schedule (←→) Day & No Deaths after Inoculation (-2 -1 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15)	% Mortality	% Showing Caecal Lesions at Autopsy: Right Caeca	Left Caeca	Bac.	Av. Indiv. Gain of Survivors
11	Stovarsol	0.75	9	903	←→ 1	11	0	0	100	537
	Stovarsol Vits.	0.75	10	977	←→ 1	10	0	0	100	339
	Vitamins alone	See Text	9	810	←→ 1-3→ 2	67	83	83	17	488
	Vioform	1.00	12	989	←→	0	0	0	0	462
	Infected Controls	None	10	884	3 4 2 1	100	100	100	0	---
12	Stovarsol	0.75	20	1664	←→ 1-2→ 1 2 1	35	0	28	72	192
	Tryparsamide	0.75	20	1523	←→ 2→3 1 3 1 1	55	0	9	91	140
	Vioform	1.00	20	1381	←→ 3-2→2 3 1 1	60	8	17	83	354
	Infected Controls	None	20	1685	2 7 7 3 1	100	85	95	5	---
	Stovarsol Controls	0.75	20	1457	←→	---	---	---	---	362
	Tryparsamide Contrs.	0.75	20	1413	←→	---	---	---	---	390
	Vioform Controls	1.00	20	1410	←→	---	---	---	---	320
	Normal Controls	None	20	1477		---	---	---	---	350
13	Stovarsol	0.75	20	1840	←→ 1 1 1 2	25	0	0	100	459
	Stovarsol	0.75	20	1901	←→ 6-4-7-2→ 1	100	80	100	0	---
	Sulfaquinoxaline	0.03¼	20	2025	←→ 1-2-2-2→6 1	70	14	86	14	440
	Infected Controls	None	19	1829	3 5 5 3 2	95	77	100	0	---
14	Stovarsol	1.25	20	2003	←→ 1	5	0	0	100	508
	Stovarsol	0.30	20	2072	←→ 1	5	0	0	100	568
	Vioform	1.00	20	1991	←→ 2-2→1 2 2	45	22	67	33	562
	Infected Controls	None	20	1896	5 10 4 1	100	95	100	0	---
	Stovarsol Controls	1.25	10	1976	←→	---	---	---	---	440
	Normal Controls	None	10	1922		---	---	---	---	428
15	Stovarsol	0.10	20	2185	←→ 2 4 2	40	0	50	50	317
	Stovarsol	0.10	20	2172	←→ 2 3 2	35	0	14	86	288
	Stovarsol	0.20	20	2375	←→ 1 6 1 3 1	60	0	17	83	171
	Stovarsol	0.30	20	2230	←→ 1 2 3 4 1	55	0	27	73	105
	Stovarsol	0.75	20	2332	←→ 1 3 1 1	30	0	17	83	478
	Infected Controls	None	20	1861	1 4 8 3 3 1	100	85	100	0	---
	Normal Controls	None	10	2403		0	---	---	---	343

dying as early as the fifth day or as late as the twelfth day.

In a comparison of the three drugs used in experiment 12 (stovarsol, tryparsamide, and vioform), the arsenicals were more effective in preventing involvement of the cecums. The birds in the vioform-treated group had lesions in 8 per cent of the right cecums, while those on the arsenicals were negative. In general, the degree of cecal involvement seemed to indicate the degree of protozoacidal activity of the drug in the gut. However, all birds dying of histomoniasis in these trials had marked liver lesions regardless of cecal damage. This fact seems to indicate early penetration of the cecal wall by Histomonas organisms with subsequent multiplication in the liver.

In a previous experiment, in which tryparsamide was administered, intravenously, greater protection was afforded when given early than when given late. It seemed desirable, therefore, to try early and late oral therapy with a drug known to possess some activity against the disease.

In experiment 13, stovarsol was given at a 0.75 per cent level in the feed to one group beginning two days before infection and to another group beginning three days after infection. All the birds in the last group were dead twelve days after infection, while only 25 per cent of the birds receiving the drug early died. Correspondingly, 100 per cent of those on early therapy had no cecal lesions and the late group was all diseased, as was the control group.

Sulfaquinoxaline was also included in this experiment at the low level of 0.034 per cent for twelve days, beginning two days prior to infection. The degree of cecal involvement and the 70 per cent mortality indicate some therapeutic value.

Since the preceding experiments afforded, to some extent, general information on the behavior of stovarsol and tryparsamide, it was desirable to examine one drug more critically. This would afford a good opportunity to try drugs of unknown activity by comparison with a drug of known activity.

The results of previous experiments had shown that late therapy was of little value. If one drug were to be studied further, the next logical procedure was administration of different concentrations in the feed. For this work, stovarsol was selected and given to groups of 20 birds each at levels of 0.30 and 1.25 per cent for ten days, beginning two days prior to infection. Vioform* was included in this trial for further evaluation, since DeVolt[3] obtained encouraging results from its use.

Both levels of stovarsol resulted in 5 per cent mortality and all cecums were negative. The group on 0.3 per cent appeared much brighter during the experiment than did the group on 1.25 per cent stovarsol. Also, yellow droppings were less frequent. The growth curves, except for vioform, for this experiment are shown in figure 3, but again the true picture is difficult to show graphically. The 1.25 per cent drug control birds showed an increased weight from the seventh to the ninth day; then the weights showed a tendency to level off. This rather sharp increase in weight may have been due to a tonic effect of the arsenical.

The vioform-treated poults during this trial were shaggy in appearance and numerous yellow droppings appeared. The mortality was 45 per cent. However, the average individual weight gains of the survivors did not indicate these conditions.

The results of experiment 14 indicate no better protection would be afforded by higher concentrations of stovarsol, since the same results were obtained at 0.3 and 1.25 per cent levels. If the upper limits were reached, it then seemed desirable to find the minimum effective level. To obtain this information, levels of 0.1, 0.2, 0.3, and 0.75 per cent were chosen. All medications were extended over a five-day period, except a second group on 0.1 per cent which was maintained ten days. This was done to study any beneficial effect over a longer period. The 0.75 per cent level was included since the effects of this concentration were better understood from previous trials.

The results seem to indicate that the minimum effective level was not reached at 0.1 per cent. There were no marked differences in mortality, although the group on 0.75 per cent appeared in much better condition during the trial, with average individual weight gains appreciably better.

The birds in this experiment were inoculated with what was believed to be a very virulent or dense culture. In other words, the control group might have shown a 100 per cent mortality with a smaller inoculum. This belief is based on the behavior of the birds receiving 0.75 per cent stovarsol. In general, this concentration of drug afforded greater protection in previous trials than was observed in this experiment. The duration of medication after infection does not seem to be a prime factor, but rather, early medication.

Immunity Studies.—During the course of the drug trials, information was obtained

*Lots of vioform used in the three experiments were not identical preparations. The preparation used in experiment 11, and for the first five days of experiment 12, was a coarse, granular appearing material with a cereal base. The preparation used in experiment 14 is known as "vioform granulate" and is much finer. The turkeys appeared to eat the two preparations at equal rates.

TABLE 5—Challenging of Portion of Recovered Turkeys

Exp. (No.)	Previous drug and experiment No.		Mortality on previous experiments (%)	No. days after 1st inoculation	No. showing previous wt. loss	No. poults challenged	Day and No. deaths after the challenging inoculation 5	6	7	8	9	10	11	12	13	14	15	Mortality (%)
18	Vioform	Exp. 11	0	15	5	12		2	4	1	2	2						92
	Stovarsol + Vit.	Exp. 11	10	15	4	8					1	1		1		1	1	62
	Stovarsol	Exp. 11	11	15	4	8					1	1					1	62
	Vitamins	Exp. 11	66	15	1	3					1				1			66
					Controls	10			3	4	2	1						100
19	Vioform	Exp. 12	60	23	3	4		1		2								75
	Tryparsamide	Exp. 12	55	23	4	4		1										25
	Stovarsol	Exp. 12	35	23	2	3		2										66
					Controls	7		2	1	2	2							109
20	Stovarsol	Exp. 13	25	18	3	8	2	1	3		1	1						100
	Sulfaquinoxaline	Exp. 13	70	18	1	3		1	2									100
					Controls	7	1	2	3	1								100
21	Miscellaneous drugs		50	18	...	13		1	2	2	1	2						61

No. challenged birds showing previous weight loss, 50 per cent; mortality in challenged birds, 72 per cent.

TABLE 6—Autopsy Findings of Portion of Recovered Birds

Exp. (No.)	Previous drug and experiment No.		Mortality on previous experiments (%)	No. poults autopsied	No. showing previous wt. loss	Days after inoculation autopsied	No. showing healing liver lesions	No. showing gross cecal lesions
19a	Tryparsamide	Exp. 12	55	5	5	23	2	1
	Stovarsol	Exp. 12	35	8	7	23	4	0
	Vioform	Exp. 12	60	3	1	23	1	0
20a	Stovarsol	Exp. 13	25	7	4	18	4	2
	Sulfaquinoxaline	Exp. 13	70	3	1	18	1	1
22	Stovarsol 0.2%	Exp. 14	5	8	2	20	3	0
	Stovarsol 1.25%	Exp. 14	5	8	4	20	3	4

which indicated medicated turkeys, surviving an experiment, were not immune. To determine if this were true, several systematic experiments were designed to obtain information on the problem of immunity, in addition to the findings of Tyzzer.[4] This investigator obtained variable periods of immunity by the use of artificially cultured Histomonas organisms. Also, turkeys subjected to constant exposure, developed immunity.

The survivors of an experiment were divided into two equal groups, as nearly alike as possible. Each group then contained the same number of birds which had lost weight or showed symptoms during a therapy trial. The plan was to challenge one group and autopsy the other. By autopsying one group, information could be obtained, on a comparative basis, as to the severity of lesions produced in the challenged group.

The results of experiments 19 and 20 (*see* table 5) seem to indicate that little immunity was obtained from the disease when the birds were medicated with the drugs studied. The data presented are too limited to draw definite conclusions, but they do indicate very little immunity is produced even in survivor birds of groups showing high mortality. Vioform in experiment 18 prevented any mortalities, while the sulfaquinoxaline group in experiment 20 suffered a 70 per cent loss; the mortalities from the challenging inoculations were similar. Of the total number of recovered turkeys challenged, 50 per cent showed weight loss on the previous experiment. The mortality in this same group after challenging was 72 per cent as compared to 100 per cent in the infected controls. (The control birds in the challenging experiments were known to be susceptible poults.)

At autopsy, the results (*see* table 6) reveal that not all birds losing weight or showing symptoms have cecal or liver lesions. The birds were autopsied eighteen to twenty-three days after infection, the lesions found were healing rapidly. There is little doubt that some of the smaller lesions had already healed and were not grossly visible at the time of autopsy. At autopsy of 42 poults, 23 showed previous weight loss. Of the 23 showing previous weight loss, 19 (84%) had gross liver lesions visible at autopsy.

CONCLUSIONS

To properly evaluate a drug, the repetition of an experiment is of the utmost importance. Even at best, in any biologic study, exact repetition of all conditions is difficult. For example, age and weight, rapid and sudden temperature and weather changes are important factors which need to be controlled or obviated in maintaining standard conditions.

A satisfactory explanation of the results obtained in experiments 4, 5, and 10 is difficult. The high protection afforded by stovarsol in experiment 5 may have been due to chance; otherwise, the results should compare closely with those obtained in experiment 4. The high mortality obtained in experiment 10, in the use of 0.75 per cent stovarsol and tryparsamide, offers more opportunity for an explanation. First, the birds were much lighter in weight, and second, control mortality was higher than in experiments 4 and 5. These factors are believed more important than the difference in drug concentration and duration of medication in these experiments.

The value of early medication was demonstrated in figure 1 and substantiated in experiment 13. This finding indicates that the flagellated form of the causative organism is more sensitive to therapy than the ameboid form found in the tissues and/or the variation is due to drug concentration in the different tissues affected.

The slight degree of cecal damage observed in medicated groups having low mortality, as compared to marked involvement in the control groups showing high mortality, is believed to be a consistent measure of drug activity. No explanation is offered as to why the left cecum is involved more frequently than the right. Regardless of the amount of cecal involvement present, the life of the turkey apparently depends, to a great extent, on the degree of liver damage.

The information obtained by challenging recovered turkeys demonstrated rather conclusively that little immunity was obtained from experimental infection, if the birds recovered as a result of drug therapy. If any immunity at all is to be acquired, a turkey must be markedly ill, almost to the point of death, and then recover. Such birds were found to be immune when repeated injections of inoculum were given several weeks apart. The belief that this protection, when present, is due to local tissue immunity rather than humoral is based on the failure of immune blood to protect susceptible turkeys.

Stovarsol, tryparsamide, and vioform, in that order, appear to be effective in reducing mortalities due to histomoniasis. Sulfaquinoxaline and iodine vermicide afforded slight protection. Sulfamerazine, sulfaguanidine, and streptomycin, anthiomaline, dimidium bromide, pentamidine, sodium fluoride, Eagle's compound, and neoarsphenamine did not appear to show any therapeutic value by the method used.

The challenging of medicated survivors of an experiment resulted in death of 72 per cent. Fifty per cent of these birds showed weight loss in a previous experiment. Autopsy of survivor birds showed 84 per cent of those that lost weight had healing liver lesions twenty days after infection.

Application of these observations and results of these studies to field conditions is a matter of practical importance. Since the method described here was designed primarily for use as a screening procedure for testing drugs against experimentally produced histomoniasis, the next logical step is to attempt therapy under field conditions.

Under field conditions, the avenue of infection is the mouth and with supposedly fewer organisms involved, as compared with the numbers given rectally in experimental inoculation. Theoretically, these large numbers of organisms and the ensuing rapid course of the disease afford a greater test for the drug. However, under field conditions, it is possible that a bird may be subjected to repeated daily doses of the infective organisms. In the single massive dose, a drug may be given at the desired time, whereas repeated light dosage obtained in the field might necessitate constant medication, at least during the period of exposure.

SUMMARY

1) A method for determining the dose of a drug for use in the therapy of experimentally produced histomoniasis is presented.

2) The rectal injection of infected, minced liver is believed to be a satisfactory method of reproducing histomoniasis. The results of this method indicate the desirability of passing the culture serially at least three times to obtain more uniform mortality in control groups.

3) The ineffectiveness of late therapy is clearly demonstrated by the use of tryparsamide and stovarsol.

4) The variations observed in these studies suggest rather strongly the desirability of repeating an experiment at least three times before reaching any definite conclusions concerning Histomonas activity.

5) Cecal involvement may be regarded as a measure of drug activity.

6) Whole blood from solidly immune turkeys failed to protect susceptible poults.

7) Data are presented which indicate very little immunity is developed by infected treated turkeys.

8) The desirability of applying the results of these studies to field conditions is evident.

References

[1]Sautter, J. H., Pomeroy, B. S., and Roepke, M. H.: Histomoniasis (Enterohepatitis) in Turkeys. II. A Procedure for the Screening and Testing of Drugs. Am. J. Vet. Res., *11*, (1950): 115-119.

[2]Tyzzer, E. E.: Studies on Histomoniasis or "Blackhead" Infections in the Chicken and the Turkey. Am. Acad. of Arts and Sci. Proc., *69*, (1934):189-264.

[3]DeVolt, H. M., and Holst, A. P.: Preliminary Report of the Preventive Action of Vioform Against Enterohepatitis (Blackhead) of Turkeys. Poult. Sci., *27*, (1948):356-358.

Effect of Sulfaguanidine upon the Developmental Stages of *Eimeria Tenella*

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IN VIEW OF the incomplete data concerning the effect of sulfaguanidine upon the life cycle of *Eimeria tenella*, the objectives of the present study were: (1) to determine, histologically, the extent of the infection in the cecums of treated and untreated chickens, and (2) to ascertain the stage of life cycle, which was affected by the drug.

LITERATURE CITED

Although the coccidiostatic action of certain of the sulfonamides has been clearly demonstrated, the stages in the life cycle of the parasite affected by sulfaguanidine are still indefinite. Levine,[1] Farr and Allen,[2] Horton Smith,[3] and Alicata and Willet[4] have reported a decrease in output of coccidial oöcysts following treatment with sulfonamides of infected chickens and swine. Swales[5] believed that the coccidiostatic properties of sulfamerazine and sulfamethazine produced an inhibition of one or more stages in the life cycle of the parasite, probably as a consequence of a weakening or destruction of the motile merozoites before they penetrated other cells of the cecal epithelium. Recently, Farr and Wehr[6] demonstrated that sulfamethazine exerted a coccidiostatic and coccidiocidal action upon the schizonts of *E. tenella* in the cecal mucosa. Waletzky and Hughes,[7] in their study on the relative merits of the sulfanilamides, concluded that the coccidiostatic action of the drugs was dependent upon their relatively high blood level, and they ascribed little importance to the drug concentration in the intestinal tract.

MATERIALS AND METHODS

All experimental chickens were single comb, white Leghorn males, obtained as day-old chicks. They were raised in battery brooders and fed on a nutritionally adequate all-mash ration. After inoculation with oöcysts, the chickens were placed in wire-bottom metal cages in small, unconnected isolation rooms. Each room was equipped with metal cages, water, and feed cups, and a gown that was worn when the chickens were examined. After termination of each experiment, the room was thoroughly washed with hot water and the equipment was steam sterilized. These precautions have proved effective in avoiding accidental infection among experimental chickens.

The strain of *E. tenella* used in these experiments was originally isolated from a field case. Oöcysts for experimental purposes were produced by infecting young, susceptible chickens with small numbers of sporulated oöcysts. The feces were collected on the sixth to the tenth days, mixed with water, strained through gauze, and the oöcysts concentrated by centrifugation. The concentrated oöcysts were sporulated in 2.5 per cent potassium dichromate solution and stored in the refrigerator. The number of oöcysts per gram of feces of treated and untreated birds was determined by the method described by Beach.[8] The method is essentially an adaptation of the procedure for counting red blood cells employing a Rafter counting apparatus.

The methods employed for determining the sulfaguanidine concentrations in the blood and cecums were described in a previous paper.[9]

The technique used for the histologic studies of the cecums was as follows. Each chicken was killed by severing the spinal cord, and sections of the proximal, middle, and distal portions of each cecum were immediately removed. The tissues were fixed in Souza's solution, dehydrated in dioxane, embedded in paraffin, sectioned serially, and stained with Heidenhain's iron hematoxylin. Mallory's and Pollak's polychromatic stains were also employed, but were not superior to the iron hematoxylin.

EXPERIMENTAL PROCEDURE

Two amounts of sulfaguanidine were administered in the feed: A 2.0 per cent mash, because of its pronounced coccidiostatic action, and a 0.5 per cent mash, because of incomplete arrest of the course of the disease as had been determined in other experiments. An infective dose of 100,000 sporulated oöcysts was chosen because, in previous trials with this culture, this number of oöcysts had produced only a moderately severe form of the disease.

In the first experiment, 36 chickens, 69-days old, were inoculated orally with *E. tenella*. Immediately following inoculation, 18 chickens were placed upon a mash containing 2.0 per cent sulfaguanidine and 18 were left on the regular mash. Beginning forty-eight hours after the experiment was started, 2 chickens from each of the treated and untreated groups were sacrificed daily and the following procedure employed: (1) The symptons and au-

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The sulfaguanidine used in the experiments was supplied by the Lederle Laboratories, Pearl River, N. Y.

topsy findings were recorded; (2) the cecal contents were removed for determination of their sulfaguanidine concentration; (3) sections of the cecums were taken for histologic study; and (4) beginning with the sixth day, determinations were made of the number of oöcysts per gram of feces passed during a 24-hour period preceding the sacrifice of the bird. Three uninoculated chickens, fed the regular mash, were held as controls. They were sacrificed on the tenth day, were autopsied, and sections were made of their cecums.

The general procedure of the second experiment did not differ from that of the first trial, except that the chickens were 55-days old and were fed a 0.5 per cent sulfaguanidine mash.

RESULTS AND DISCUSSION

The results of these trials are shown in tables 1 and 2. The figures used in columns 4 and 6 are comparisons of the extent of infection of the treated and corresponding untreated chickens which were sacrificed on the same day. They are not directly related to the extent of infection on any other day. These comparisons were made on the basis of the number of parasitized cells seen and the extent of the pathology of the surrounding tissues.

Symptoms and Autopsy Lesions.—The 2.0 per cent sulfaguanidine mash exhibited an anticoccidial action so marked that no symptoms of coccidiosis were evident. Upon autopsy, only 1 (bird 7) of the 18 treated chickens showed a grayish white focal nodule about 1 mm. in diameter in one cecum, otherwise no gross lesions were present. No oöcysts were recovered from the droppings of the treated group during the ten-day trial. The untreated chickens, on the other hand, showed typical symptoms of a moderate-to-severe form of the disease, beginning on the fifth day with a progressive improvement in the remaining birds by the eighth day. Severe gross lesions of cecal coccidiosis were found in the birds sacrificed on the fifth to ninth days. The 2 chickens killed on the tenth day of the experiment presented considerably milder lesions with some evidence of healing.

When the 0.5 per cent sulfaguanidine mash was used, the treated chickens developed clinical coccidiosis but in a milder form than observed in the corresponding untreated controls. The gross pathologic lesions in the treated chickens on autopsy simulated those found in the untreated birds. However, the number of petechiae and foci of coccidia were considerably smaller, and the hypertrophy of the walls and mucous membranes was less pronounced.

No symptoms were observed in the 6 uninoculated control chickens. An autopsy and microscopic examination of the cecums showed no evidence of a coccidial infection.

Effect upon the Sporozoites.—Neither the 2.0 per cent nor the 0.5 per cent sulfaguanidine mash appeared to have a harmful effect on the sporozoites. This was shown by the

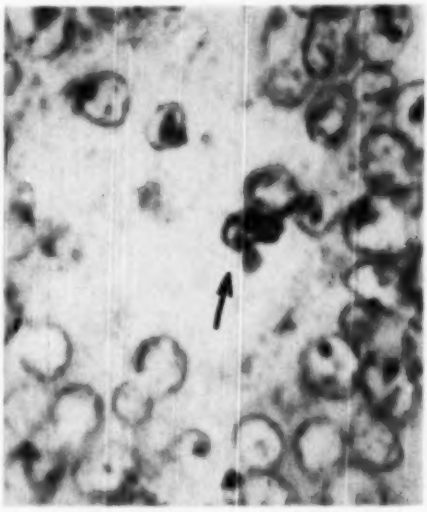

Fig. 1—Early developmental stage of a first generation schizont with its characteristic granule in the epithelium of the cecum. Chicken sacrificed forty-eight hours following inoculation with 100,000 sporulated oöcysts of *Eimeria tenella* and continuously fed a mash containing 2.0 per cent sulfaguanidine. Fe hematoxylin. x 2300.

fact that a sufficient number of sporozoites survived in the presence of the drug in the intestinal tract to invade the epithelium and produce nearly as many schizonts in the treated birds, during the second and third days, as developed in the untreated birds. (*See* tables 1 and 2.) Further development of the parasite was definitely retarded by the 2 per cent sulfaguanidine mash. Immature first generation schizonts in stages of early development, as shown in figure 1, were found in the epithelial cells of all of the chickens treated with 2 per cent sulfaguanidine mash. By contrast, the majority of the parasites continued their development in the chickens treated with 0.5 per cent sulfaguanidine mash (table 2). Among birds receiving the lower concentration of sulfaguanidine, it appeared that at least a few first generation schizonts were retarded in their development because such schizonts were found in the epithelium in the absence of other forms of the parasite in those birds

which were sacrificed on the tenth day. Tyzzer[10] observed that all the sporozoites liberated in the intestinal tract do not penetrate the epithelium simultaneously, and that the phases of the life cycle of the parasite are not equally developed at any given time. It is doubtful, however, that

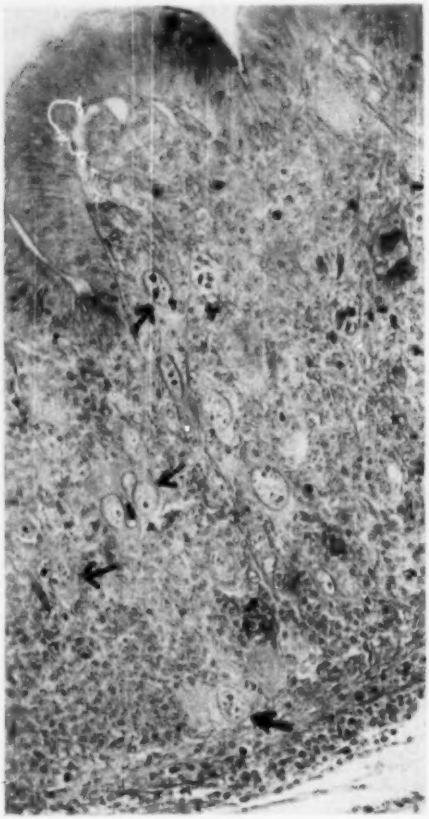

Fig. 2—Degenerated second generation schizonts in the lamina propria of the cecal mucosa seven days following inoculation with 100,000 sporulated oöcysts of *Eimeria tenella* and treatment with 2.0 per cent sulfaguanidine mash. Heidenhain's Fe hematoxylin. x 280.

the lag in development of the parasite normally extends to the tenth day, and therefore, it is more reasonable to assume that the sporozoites penetrated the epithelial cells and further growth was inhibited by the static action of the drug. This assumption is further substantiated by the report of Farr and Wehr.[11] They demonstrated that as long as sulfaguanidine was given in sufficient amounts to chickens inoculated with *E. tenella*, the disease could be controlled, but, following the withdrawal of the medicated mashes, bloody droppings reappeared, and the oöcyst output gradually increased.

Effect upon Schizogony.—Sulfaguanidine apparently had a marked anticoccidial action upon the asexual phase of the life cycle. All of the chickens treated with 2.0 per cent sulfaguanidine mash, which were sacrificed on the fourth to the tenth days after inoculation, showed relatively few parasites in the tissues. Microscopically, immature first generation schizonts were found in all of the chickens. No evidence of degeneration of the young trophozoites was found. Three chickens, namely, 1 killed on the fifth day and 2 killed on the seventh day (table 1), showed, in addition to the

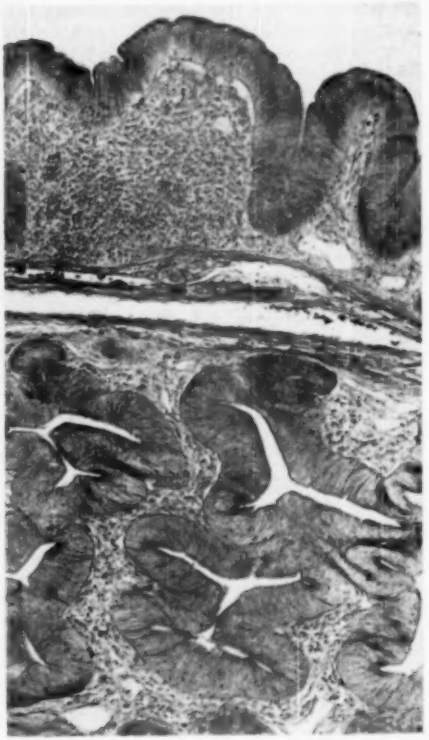

Fig. 3—Intact cecal mucosa of a chicken eight days after inoculation with 100,000 sporulated oöcysts of *Eimeria tenella* and continuously treated with a 2.0 per cent sulfaguanidine mash. Note absence of second generation schizonts and gametocytes. Fe hematoxylin. x 135.

immature first generation schizonts, foci containing second generation schizonts in the lamina propria of the villi and interglandular spaces (fig. 2). The second generation schizonts showed evidence of degeneration. The cytoplasm of the trophozoites lost its cellular detail, became vacuolated, or condensed into a homogeneous mass containing a variable number of relatively large, dark-staining granules. The schizonts were surrounded by hyaline. The areas in which these degenerating schizonts were found were infiltrated with eosinophils and red blood cells, but the extent of the inflammatory response and the microscopic lesions which were found on autopsy were relatively insignificant when compared with the respective untreated controls. Sulfaguanidine, given at the rate of 2.0 per cent in the mash, suppressed the development of some of the first generation schizonts, but a few schizonts were able to mature and liberate merozoites to form second generation schizonts. The latter were destroyed by the drug during their development in the tissues (fig. 2).

Fig. 4—Glandular portion of a cecum containing gametocytes and oöcysts in control chicken on the eighth day following inoculation with 100,000 sporulated oöcysts of *Eimeria tenella*. Heidenhain's Fe hematoxylin. x 350.

Infected chickens, given 0.5 per cent sulfaguanidine mash, presented, microscopically, all stages of the life cycle of the parasite in the chickens sacrificed between the fourth and eighth days. Although the severity of the histopathologic response of the host to the parasite was considerably milder than in the untreated controls, no evidence of degeneration of the first and second generation schizonts was seen. However, the chickens which were examined on the fourth and fifth days presented fewer second generation schizonts than the corresponding controls (table 2). In a previous experiment,[12] as well as in the work reported by Farr and Allen,[2] it was shown that the oöcyst output was considerably reduced when treatment of infected chickens was delayed until the third, fourth, or fifth days following inoculation. At that stage in the life cycle, the merozoites are being liberated from the schizonts in great numbers. These findings further indicated that the effect of 0.5 per cent sulfaguanidine mash is to weaken or destroy free merozoites, thereby limiting the number of schizonts formed.

Effect upon the Gametocytes.—The coccidiostatic and coccidiocidal properties of sulfaguanidine upon the schizonts and merozoites, which were liberated from surviving schizonts, precluded gametogony in the chickens which were given the 2 per cent sulfaguanidine mash. Figure 3 shows the intact cecal mucosa, free of gametocytes, of a chicken which was sacrificed on the eighth day. By comparison, figure 4 illustrates the large number of gametocytes in the cecal mucosa of the corresponding control.

The data in table 2 show that birds given a mash containing 0.5 per cent sulfaguanidine produced fewer oöcysts than the corresponding untreated chickens. Further-

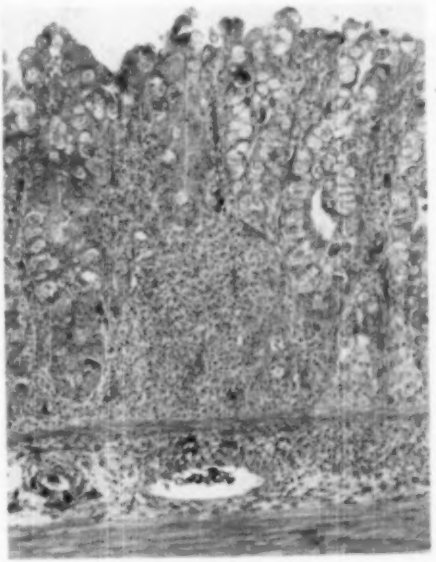

Fig. 5—Cecal mucosa showing gametocytes and oöcysts in the epithelium of control chicken on the tenth day following inoculation with 100,000 sporulated oöcysts of *Eimeria tenella*. Fe hematoxylin. x 80.

TABLE 1—The Effect of Sulfaguanidine upon the Life Cycle of *Eimeria Tenella* in Chickens Inoculated with 100,00 Sporulated Oöcysts and Immediately Fed *Ad Libitum* a Mash Containing 2.0 Per Cent of the Drug*

Day sacrificed	Bird (No.)	Treated			Untreated	
		S.G. conc. (mg. per 100 Gm. cecal contents)	Histopathology	Oöcyst discharged per Gm. feces	Histopathology	Oöcyst discharged per Gm. feces
2nd	1	2,260	1+ Immature schiz.	Neg.	3+ Immature schiz.	Neg.
	2	1,475	3+ Immature schiz.		3+ Immature schiz.	
3rd	3	4,105	2+ Immature schiz.	Neg.	2+ Maturing schiz.	Neg.
	4	2,595	3+ Immature schiz.		3+ Maturing schiz.	
4th	5	1,615	1+ Immature schiz.	Neg.	3+ 1st and 2nd gen. schiz.	Neg.
	6	4,070	1+ Immature schiz.		3+ 1st and 2nd gen. schiz.	
5th	7	2,285	2+ Immature 1st and few maturing 2nd gen. schiz.	Neg.	2+ 1st and 2nd gen. schiz.	Neg.
	8	1,040	1+ Immature schiz.		2+ 1st and 2nd gen schiz., begin. gamet.	
6th	9	1,700	1+ Immature schiz.	Neg.	4+ Mature schiz., gamet., ed., cell infilt., hem.	Neg.
	10	2,190	1+ Immature schiz.		4+ Mature schiz., gamet., ed., cell infilt., hem.	
7th	11	2,090	1+ Immature 1st and few maturing 2nd gen. schiz.	Neg.	4+ Mature schiz., gamet., ed., cell infilt., hem.	691,250
	12	1,460	2+ Immature 1st gen. and few mature 2nd gen. schiz., sl. cell response.		4+ Mature schiz., gamet., ed., cell infilt., hem.	
8th	13	2,450	1+ Immature schiz.	Neg.	4+ Mature schiz., gamet., ed., cell infilt., hem.	512,500
	14	2,180	1+ Immature schiz.		3+ Gamet., predom. oöcysts, mkd. ed., cell infilt.	
9th	15	2,335	1+ Immature schiz.	Neg.	3+ Gamet., predom. oöcysts, ed.	88,481
	16	2,150	1+ Immature schiz.		4+ Gamet., predom. oöcysts, ed.	
10th	17	2,585	1+ Immature schiz.	Neg.	3+ Gamet., oöcysts, active prolif. and regen.	41,391
	18	2,935	1+ Immature schiz.		2+ Gamet., oöcysts, active prolif. and regen.	

*ed. = edema; gamet. = gametocytes; gen. = generation; hem. = hemorrhage; infilt. = infiltration; mkd. = marked; predom. = predominate; schiz. = schizonts; s.g. = sulfaguanidine; sl. = slight.

TABLE 2—The Effect of Sulfaguanidine upon the Life Cycle of *Eimeria Tenella* in Chickens Inoculated with 100,000 Sporulated Oöcysts and Immediately Fed *ad Libitum* a Mash Containing 0.5 Per Cent of the Drug*

		Treated			Untreated	
Day sacrificed	Bird (No.)	S.G. conc. (mg. per 100 Gm. cecal contents)	Histopathology	Oöcyst discharged per Gm. feces	Histopathology	Oöcyst discharged per Gm. feces
2nd	1	416	3+ Immature schiz.	Neg.	3+ Immature schiz.	Neg.
	2	680	2+ Immature schiz.		2+ Immature schiz.	
3rd	3	512	3+ Maturing schiz.	Neg.	3+ Maturing schiz.	Neg.
	4	872	2+ Maturing schiz.		2+ Maturing schiz.	
4th	5	168	3+ 1st and 2nd gen. schiz.	Neg.	4+ 1st and 2nd gen. schiz., ed., hem., cell infilt.	Neg.
	6	416	3+ 1st and 2nd gen. schiz.		4+ 1st and 2nd gen. schiz., ed., hem., cell infilt.	
5th	7	60	2+ 1st and 2nd gen. schiz., begin. gamet., hem, sl. ed., cell infilt.	Neg.	4+ 1st and 2nd gen. schiz., begin. gamet., slough., ed., hem., cell infilt.	Neg.
	8	72	3+ 1st and 2nd gen. schiz., begin. gamet., hem., sl. ed., cell infilt.		3+ 1st and 2nd gen. schiz., begin. gamet., slough., ed., hem., cell infilt.	
6th	9	450	3+ 2nd gen. schiz. gamet. predom. ed., hem., cell infilt.	1,214	Died, no sections.	57,816
	10	200	3+ 2nd gen. schiz. gamet. predom. ed., hem., cell infilt.		4+ Schiz., gamet., sloughing, ed., hem. cell infilt.	
7th	11	360	3+ Schiz., gamet., oöcysts, ed., hem., cell infilt.	400,700	4+ Schiz., gamet., oöcysts, slough., ed., hem., cell infilt.	532,170
	12	660	4+ Schiz., gamet., oöcysts, ed., hem., cell infilt.		4+ Schiz., gamet., oöcysts, slough., ed., hem., cell infilt.	
8th	13	235	4+ Schiz., gamet., oöcysts, ed., hem., cell infilt.	2,465	4+ Gamet., oöcysts, mkd. hyperplasia, most mucosa sloughed and regen.	17,739
	14	240	4+ Schiz., gamet., oöcysts, ed., hem., cell infilt.		Died 8th day, no sections.	
9th	15	150	1+ Gamet.	Neg.	4+ Gamet., oöcysts, sl. ed.	192,601
	16	725	2+ Gamet., sl. ed.		3+ Gamet., oöcysts, sl. ed.	
10th	17	90	1+ Immature schiz.	100	3+ Gamet., oöcysts.	89,152
	18	765	1+ Immature schiz.		2+ Gamet., oöcysts.	

*ed. = edema; gamet. = gametocytes; gen. = generation; hem. = hemorrhage; infilt. = infiltration; mkd. = marked; predom. = predominate; schiz. = schizonts; s.g. = sulfaguanidine; sl. = slight.

more, upon autopsy on the ninth and tenth days following inoculation, the cecal lesions were considerably milder and, microscopically, the gametocytes and oöcysts were either absent or present in conspicuously smaller numbers than in the corresponding untreated chickens. The fact that the feces of the treated birds contained only 100 oöcysts per gram indicated that only a few of the gametocytes reached maturity to form oöcysts and therefore that the life cycle of *E. tenella* was interrupted by the action of the drug on schizogony.

Sulfaguanidine Concentrations in the Cecums.—The average concentration of sulfaguanidine in the cecums at the time of sacrifice of the chickens given 2.0 per cent of the drug in the mash was 2,305 mg. per 100 Gm. of cecal contents with a range varying from 1,040 to 4,105 mg. per 100 Gm. Chickens given the 0.5 per cent sulfaguanidine mash showed a greater fluctuation in the amount of drug in the cecal contents, the average being 392.83 mg. per 100 Gm. of material with the range varying from 60 to 872 mg. per 100 Gm. of feces. The lowest sulfaguanidine concentrations in the cecums of chickens which received the 0.5 per cent sulfaguanidine mash were found on the fifth day, which is the period when the parasites have caused the most severe damage. It is possible that the condition of the individual birds may have some influence upon the amount of drug consumed and its accumulation in the cecums.

No blood-sulfaguanidine levels were determined. However, in another experiment, the average drug concentration in the blood of normal chickens given 0.5 per cent and 2.0 per cent sulfaguanidine mash was found to be 2.60± 1.31 and 12.87± 6.21 mg. per 100 cc. of blood, respectively.[9]

SUMMARY AND CONCLUSIONS

1) This paper reports the results of experiments to determine the stage in the life cycle of the protozoon which is affected by sulfaguanidine. In this work, groups of chickens were inoculated with sporulated oöcysts of *Eimeria tenella* and, immediately following, were either continuously fed mash containing 2.0 per cent or 0.5 per cent sulfaguanidine or left untreated as controls. Beginning forty-eight hours after inoculation, 2 chicks from each of the treated and untreated groups were sacrificed daily to permit histopathologic examination of the cecums and sulfaguanidine determination of their cecal contents.

2) Mashes containing 2.0 per cent and 0.5 per cent sulfaguanidine fed *ad libitum* had no demonstrable effect upon the sporozoites.

3) Sulfaguanidine in the mash, given at the rate of either 2.0 per cent or 0.5 per cent showed evidence of coccidiostasis upon the first generation schizonts.

4) The 2.0 per cent sulfaguanidine mash was coccidiocidal to the second generation schizonts in the lamina propria. No evidence of degeneration of schizonts was found in chickens given 0.5 per cent sulfaguanidine mash, but the asexual cycle was assumed to be indirectly affected by the action of the drug upon the merozoites in the lumen of the cecums. This limited the extent of further parasitism of the tissues.

5) These data suggest that sulfaguanidine exerts coccidiostatic and coccidiocidal actions upon the schizogonous stages of *E. tenella;* the degree of the effectiveness was influenced by the amount of drug administered.

References

[1]Levine, P. P.: The Effect of Sulfanilamide on the Course of Experimental Avian Coccidiosis. Cornell Vet., *29*, (1939): 309.

[2]Farr, M. M. and Allen, R. W.: Sulfaguanidine Feeding as a Control Measure for Cecal Coccidiosis of Chickens. J. A. V. M. A., *100*, (1942): 47.

[3]Horton-Smith, C.: Sulfaguanidine in Avian Coccidiosis. Vet. Rec., *54*, (1942): 259.

[4]Alicata, J. E. and Willet, E. L.: Observations on the Prophylactic and Curative Value of Sulfaguanidine in Swine Coccidiosis. Am. J. Vet. Res., *7*, (1946): 24.

[5]Swales, E. E.: On the Chemotherapy of Cecal Coccidiosis (*Eimeria Tenella*) of Chickens. Canad. J. Res., *22*, (1944): 131.

[6]Farr, M. M., Wehr, E. E.: Developmental Stages in the Life Cycle of *Eimeria Tenella* Affected by Sulfamethazine Treatment. Proc. Helminth. Soc., Washington, *14*, (1947): 12-20.

[7]Waletzky, E. and Hughes, C. O.: The Relative Activity of Sulfanilamides and Other Compounds in Avian Coccidiosis (*Eimeria Tenella*). Am. J. Vet. Res., *7*, (1946): 365.

[8]Beach, J. R.: A Rapid Method for Quantitative Counts of Coccidial Oöcysts in Chicken Feces. Cornell Vet., *34*, (1943): 308.

[9]Bankowski, R. A.: Some Pharmacologic Aspects of Sulfaguanidine in Normal Chickens. Am. J. Vet. Res., *9*, (1948): 85.

[10]Tyzzer, E. E.: Coccidiosis in Gallinaceous Birds. Am. J. Hyg., *10*, (1929): 269.

[11]Wehr, E. E. and Farr, M. M.: Effect of Sulfaguanidine on the Course of Infection in Chickens with *Eimeria Tenella*. J. Parasitol., *31*, (1945): 359.

[12]Recent Studies of Poultry Disease at the University of California. New Laid News, *19*, (1942): 27.

A Whole Blood, Rapid Agglutination Test for Avian Tuberculosis—A Preliminary Report

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THE PRESENT method of testing chickens for tuberculosis requires that each flock be handled twice. Avian tuberculin must be carefully injected intracutaneously in the wattle and each chicken must be handled again forty-eight hours later in order to observe the results.* Obviously, this limits the number of flocks that can be tested by 1 veterinarian in a given length of time and, furthermore, may occasion some objections on the part of the caretaker who must arrange for confining the flock and handling each chicken on two successive occasions forty-eight hours apart. In contrast, the testing of chickens for pullorum disease by the whole blood, rapid agglutination test is a less time-consuming procedure. The birds are confined and tested at once and reactors are disposed of immediately without a second handling.

The possibility of using a whole blood, rapid agglutination test for avian tuberculosis was suggested by the work of Moses, Feldman, and Mann,[1] who described a serum rapid agglutination test as an aid in the diagnosis of tuberculosis in pigeons and chickens. These workers succeeded in preparing a stable, concentrated suspension of avian tubercle bacilli which could be used as an antigen for the rapid plate agglutination test with serum. Comparisons of the tuberculin tests, necropsy findings, and serum rapid agglutination test on a flock of 50 chickens revealed that, although the agglutination test may fail to disclose minimal tuberculous infections, it may have a reliability comparable to that of the tuberculin test.

The serum rapid agglutination test for avian tuberculosis may be of value for laboratory use, but it is obviously of no advantage over the tuberculin test for field testing when one considers the time required to collect blood specimens from each bird and to separate the serum before doing the test. Furthermore, such a procedure requires that every bird be banded for identification and that each bird be handled a second time to identify reactors.

It was thought desirable to explore the possibility of using a concentrated antigen of avian tubercle bacilli for a rapid, whole blood, agglutination test that could be done by mixing on a glass plate 1 drop of fresh whole blood with 1 drop of antigen and recording the results of the test within one minute. If successful, such a test could be done at the same time as the whole blood, rapid plate agglutination test for pullorum disease. Each bird could be held or otherwise confined until completion of the test.

METHODS

Preparation of Antigen.—In this exploratory investigation, we were not immediately concerned with studies of procedures for preparing concentrated antigen of avian tubercle bacilli. The main problem at the outset was to select cultures of avian tubercle bacilli which would produce uniform and stable suspensions. Forty strains of avian tubercle bacilli were available from which to select cultures which could be grown in relatively large quantities and which would not show autoagglutination. Fifteen were stock cultures of avian tubercle bacilli that had been maintained on egg-yolk agar medium for two to five years, and 25 had been recently isolated from chickens obtained from farm flocks.

Proskauer and Beck liquid synthetic medium was used for growing the cultures because it is routinely prepared in our laboratory[2] and we had previously observed that avian tubercle bacilli will produce a uniformly turbid growth in this medium without granular clump formation.

The liquid medium was placed in 250-ml. Erlenmeyer flasks in 100-ml. amounts. Two flasks were used for each of the 40 strains of avian tubercle bacilli. The medium was inoculated by rubbing a loopful of culture on the inner wall of the flask at the level of the liquid so that the bacilli would disperse evenly in the medium. After three weeks of incubation at 37 C., the growth was removed by centrifu-

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*For a detailed discussion on the tuberculin test in chickens see the monograph by W. H. Feldman, Avian Tuberculosis Infections, Williams & Wilkins Co., Baltimore, Md., 1938.

gation and washed three times in sterile water containing 0.5 per cent phenol. After the third washing, the bacilli were suspended at a concentration of 10 per cent by volume in physiologic saline solution containing 0.5 per cent phenol. This suspension was used as the antigen for subsequent tests. Approximately, 3 to 4 ml. of antigen could be prepared from each pair of flasks.

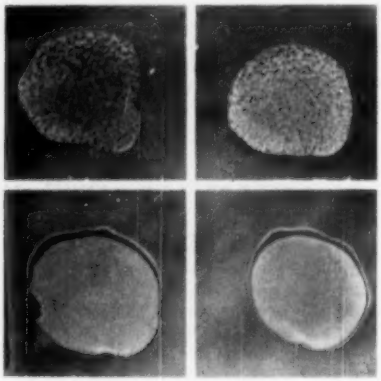

Fig. 1—Whole blood, rapid agglutination test for avian tuberculosis. The two upper tests were made with whole blood from chickens that were tuberculin-positive and were found to have lesions typical of the disease. The two lower tests were made from tuberculin-negative chickens which had no demonstrable lesions. The positive reactions were discernible in one minute. The photograph was made ten minutes later in order to show the absence of clumping in the negative tests after a relatively long period.

In addition to the 40 antigens prepared from growth on the liquid synthetic medium, 14 were prepared from growth on egg-yolk agar, which is routinely available in our laboratory.[2] The egg-yolk agar medium, which was slanted in 2-oz. prescription bottles, was inoculated by distributing 1 ml. of a suspension of avian tubercle bacilli over the surface by means of a pipette. Four bottles of egg-yolk agar were inoculated with each of the 14 strains. After three weeks of incubation, the growth was washed off the surface of the agar with physiologic saline solution containing 0.5 per cent phenol. After being washed three times, the bacilli were suspended in a 10 per cent concentration in physiologic saline solution containing 0.5 per cent phenol for subsequent use as an antigen. From 1 to 2 ml. of antigen could be prepared from the four bottles of agar.

Stability of the Antigens.—Seventeen of the 40 antigens were unstable. Drops of these antigens promptly showed autoagglutination when placed on a warmed glass plate. The bacteria rapidly clumped when drops of the antigen were mixed with either physiologic saline solution, normal horse serum, or normal chicken serum, as well as with serum from tuberculous chickens. No further work was done with the unstable antigens or the cultures from which they were prepared.

It is not known whether the phenomenon of autoagglutination is inherent in the particular strains that showed it or whether it is a result of the method of cultivation. Strains which produced unstable antigen when cultured on the liquid synthetic medium also produced autoagglutinable antigens when cultured on egg-yolk agar. The occurrence of autoagglutination was not apparently related to the age of the culture, since it was noted in the antigens prepared from six of the 15 stock cultures and in 11 of the 25 recently isolated cultures. In four instances, cultures from different chickens from the same farm yielded both stable and unstable antigens.

Further investigations must be made to determine, if possible, the causes of the autoagglutination of some strains of avian tubercle bacilli.

Specificity of the Antigens.—Each of the 23 antigens which did not show autoagglutination was tested for specificity by using rapid serum agglutination tests with 17 serums from normal chickens and 25 serums from tuberculous chickens. These 25 serums represented nine different farm flocks from which the tuberculous birds were obtained. The tests were done by mixing on a warmed glass plate 1 drop of antigen with 1 drop of serum. The presence or absence of agglutination was recorded in one minute.

Ten of the 23 antigens showed some reaction with serum from normal chickens. These reactions varied from slow and incomplete to rapid and complete with six or more of the 17 negative serums. In these tests, there was no evidence of autoagglutination in the drops of whole antigen or in the mixtures of antigen and physiologic saline solution. These ten antigens were excluded for further studies. The cause of the agglutination of ten of the otherwise stable antigens with serum from chickens that had no demonstrable evidence of tuberculosis is not explainable by the information available.

Only 13 antigens remained for further study after excluding those showing autoagglutination and those which reacted with some of the serum from presumably normal chickens. Eight of the 13 appeared to be less sensitive than the others because they were agglutinated with less than 90 per cent of the positive serums. These were discarded.

The procedures described in previous para-

graphs for selecting suitable antigens for field trials with whole blood limited the number of antigens to five from the original total of 40. Four of the five were recently isolated cultures obtained from chickens from different farms. The other culture had been maintained in our stock culture collection for approximately four years.

FIELD TRIALS

A comparison of the tuberculin test and the whole blood, rapid agglutination test was made on four farm flocks with a total of 282 chickens. The whole blood, agglutination test was used on each bird at the time of observation of the results of the tuberculin test. (Studies in a flock of 40 chickens known to be free of tuberculosis indicated that the injection of avian tuberculin in the usual manner would not result in a positive whole blood, rapid agglutination reaction when the chickens were tested forty-eight hours later.)

The whole blood, agglutination test was done by mixing on a warm plate 1 drop of blood with 1 drop of antigen and recording the results in one minute. The blood was procured from each chicken by pricking a point of the comb with an 18-gauge hypodermic needle and collecting a drop of the blood on the edge of a glass microscope slide. By means of the glass slide, the blood was immediately mixed with a drop of antigen previously placed on the glass plate. The plate was gently rocked and placed on a box warmed by two 60-watt bulbs.* Figure 1 presents two positive and two negative reactions. The clumping of the antigen is readily seen in the two positive reactions.

It was found to be impractical to test each chicken with each of the five antigens. After 24 chickens had been tested with each of the antigens, it was observed that there was no apparent difference between the antigens, and one was then arbitrarily selected for the remainder of the tests.

The results of the field tests on farm flocks are recorded in table 1. It will be noted that tuberculin-positive chickens were found in each of the first three flocks. These were farm flocks consisting of pullets and old hens which had free range of the premises. Every chicken that had a positive tuberculin reaction also had a positive agglutination reaction. In addition, there were a few chickens in each of these flocks that failed to react to tuberculin but gave a positive agglutination reaction. It is of interest that in flock 4, where there were no tuberculin reactions, there were no agglutination reactors. This flock consisted of year-old chickens which were not allowed free range of the farm.

*The tests were done with a testing box normally used for the serum rapid plate test for brucellosis.

The 14 birds that showed no tuberculin sensitivity but which were positive to the agglutination test were subjected to further study. One bird was found to have several ulcerative tuberculous lesions involving the ileocecal junction plus a large, single tubercle in the spleen. A second bird had a few small tubercles in the liver only. No grossly visible lesions were demonstrable in any of the 12 remaining birds. Portions of liver, spleen, lungs, kidneys, bone marrow, and ileocecal junction were preserved from each chicken for histologic examination. The liver and spleen were cultured for avian tubercle bacilli. Serum specimens for further agglutination tests were obtained from 9 of the 14 birds, including the 2 with visible lesions. Each of the nine serums agglutinated avian tubercle bacilli when tested in the laboratory. Five of the nine serums were tested six months later. It was found that they still agglutinated suspensions of avian tubercle bacilli. (The other four serums were not available for examination.)

Avian tubercle bacilli were isolated from the liver and spleen of the 2 chickens that had lesions demonstrable on necropsy and acid-fast bacilli were demonstrable in the microscopic sections. Histologically, the lesions were typical of spontaneous avian tuberculosis.

Cultures of the liver and spleen of the 12 chickens that had no visible lesions failed to show any growth after ninety days of incubation. No acid-fast bacilli could be demonstrated in tissue sections, nor was there any histologic evidence of tuberculosis in the sections of liver, spleen, kidneys, bone marrow, or ileocecal junction.

TABLE 1—Results of the Tuberculin Test and of the Whole Blood, Rapid Agglutination Test for Avian Tuberculosis in Four Farm Flocks of Chickens*

Flock (No.)	No. of chickens	Tuber. pos. agglut. pos.	Tuber. neg. agglut. pos.	Tuber. pos. agglut. neg.
1	91	10	8	0
2	57	30	5	0
3	90	1	1	0
4	44	0	0	0
Total	282	41	14	0

*All birds which reacted to the tuberculin test also gave a positive agglutination reaction. A few birds in each infected flock had positive agglutination reactions but negative tuberculin reactions.

COMMENT

This pilot study discloses many problems that await further investigation. Of the 40 cultures used to prepare antigens, 17 produced suspensions that agglutinated spontaneously. Such cultures should be

studied to determine the factors responsible for autoagglutination and thus establish criteria for selecting suitable strains for the production of antigens. Incidental observations in this work suggested that the age of the culture and the method of cultivation, either on solid or on liquid medium, did not affect the stability of the antigens, but the limited observations are not conclusive. No attempt was made to determine the effect of pH of the suspending solution, the comparative effects of phenol and formaldehyde as a preservative, or the effect of using dyes to color the antigen. A 10 per cent suspension was arbitrarily selected because at that concentration the clumping of the bacteria in positive serum was clearly visible. Clumping was readily seen when used with whole blood. It is obviously necessary to learn if such a heavy concentration is necessary and whether it may be responsible for prozonal inhibition of agglutination.

The fact that only five of 23 stable cultures showed a high degree of specificity when tested with known positive serum brings up the question of type-specific antigens. Is there sufficient serologic difference among strains of avian tubercle bacilli to require the use of polyvalent antigens for a whole blood, agglutination test? Rotach[4] has recently reviewed the serologic differences that exist among strains of avian tubercle bacilli and has shown that with the strains he studied there is cross agglutination in spite of the occurrence of type-specific antigens.

The role of the saprophytic acid-fast bacteria in the formation of agglutinins against avian tubercle bacilli as well as their significance in sensitizing chickens to avian tuberculin should be examined. It has been demonstrated that certain saprophytic acid-fast microorganisms found in tissues of animals are serologically related to avian tubercle bacilli.[5]

Of particular interest in this study are the 14 chickens which failed to react to tuberculin but which did react to the whole blood, agglutination test. Two had lesions typical of avian tuberculosis but neither had extensive disease such as is usually associated with anergy to tuberculin. The other 12 chickens in this group present a problem since they had no lesions that could be demonstrated grossly or histologically, nor was it possible to isolate acid-fast bacteria from their spleens or livers. It may be significant that these chickens came only from flocks where the disease was known to exist, and that no such birds were found in the one flock that was known to be free of avian tuberculosis. It is possible that these 12 chickens had been recently infected and that the lesions were too small to be demonstrated. The lesions, if present, may have been located elsewhere than the spleens or livers, which were the only organs cultured. This consideration presents the question whether agglutinins or tuberculin sensitivity appears first.

SUMMARY AND CONCLUSIONS

Concentrated antigens for a whole blood, rapid agglutination test for avian tuberculosis were prepared from 40 strains of avian tubercle bacilli grown in liquid synthetic medium. Seventeen of the 40 cultures produced antigens which could not be used because of autoagglutination. Only five of the remaining 23 antigens showed a high degree of specificity when tested with serum from tuberculous chickens and from normal chickens. A comparison of the tuberculin reaction and the whole blood, plate agglutination test was made on four farm flocks with a total of 282 birds. Forty-four reacted to the tuberculin test and each of these also gave a positive agglutination reaction. No birds had positive tuberculin reactions and negative agglutination reactions, but there were 14 in which the tuberculin reaction was negative and the agglutination reaction positive. Two of these 14 had grossly visible lesions of tuberculosis. The other 12 had no lesions demonstrable at necropsy nor was it possible to demonstrate lesions or acid-fast bacilli by histologic or cultural procedures. The chickens that reacted only to the agglutination test came from three farms where avian tuberculosis was known to exist. There were no agglutination reactors in the one flock that had no tuberculin reactors.

The observations made so far suggest that a whole blood, rapid agglutination test for avian tuberculosis can be used to detect infected flocks. The test requires less time than the tuberculin test and could be done at the same time as the whole blood test for pullorum disease. Further work is required to select suitable strains of avian tubercle bacilli for the preparation of antigens. In addition, it will be necessary to determine the optimal conditions of growth of suitable strains, the optimal concentration of the antigen, the optimal pH, and the effect of preservatives on the antigens. The specificity of the whole blood, agglutination test for avian tuberculosis must be determined by comparing it with the results of tuberculin tests, pathologic examination, and bacteriologic procedures. The data obtained in this preliminary study seem sufficiently significant to justify a more com-

prehensive investigation of a procedure that might prove useful in detecting avian tuberculous infections.

References

[1]Moses, H. E., Feldman, W. H., and Mann, F. C.: Mycobacterial Rapid Agglutination Antigens and Their Diagnostic Value in Tuberculosis of Fowl. Am. J. Vet. Res., *4*, (1943) :390-394.

[2]Youmans, G. P., and Karlson, A. G.: Streptomycin Sensitivity of Tubercle Bacilli; Studies on Recently Isolated Tubercle Bacilli and the Development of Resistance to Streptomycin in Vivo. Am. Rev. Tuberc., *55*, (1947) :529-535.

[3]Karlson, A. G., and Necunam, G. M.: Determination of Streptomycin Sensitivity of Tubercle Bacilli by Use of Egg-Yolk Agar Medium. Proc. Staff Meeting, Mayo Clin., *23*, (1948) :401-408.

[4]Rotach, F.: La classification sérologique des bacilles de la tuberculose aviaire. Schweiz. Ztschr. f. Path. u. Bakt., *10*, (supplement), (1947) :325-348.

[5]Karlson, A. G., and Feldman, W. H.: Studies on an Acid-Fast Bacterium Frequently Present in Tonsillar Tissue of Swine. J. Bact., *59*, (1940) :461-472.

Discussion

DR. SHELTON (Va.): Dr. Karlson, did you have any difficulty keeping the antigen? Did you have to break up the culture?

DR. KARLSON: The antigens used in the field studies appeared to remain stable and sensitive during storage in the refrigerator for several months. It was not necessary to break up the cultures. Unlike mammalian tubercle bacilli, avian tubercle bacilli are easily suspended in broth, water, or physiologic saline solution.

DR. H. F. SMITH (Texas): It seems that your tuberculin test missed 2 birds that the agglutination test picked up. Is there any information as to whether you have missed any or how many might have been missed by both tests?

DR. KARLSON: We do not know how many infected birds, if any, may have been missed by both tests. Further studies are required to determine whether the agglutination test will fail to detect infected birds.

Comparative Toxicity of DDT and Chlordan to Young Chicks

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IN A RECENT study of floor brooding, chicks died in large numbers when brooded on litter (bagasse) treated with a spray containing 1 lb. of DDT,* 1 lb. of chlordan,** and 1¼ oz. of lethane (B-72)† in 10 qt. of water. The rate of death rose precipitously forty-eight to seventy-two hours following exposure to the treated litter. Many chicks were hyperexcitable, trembled, and entered into clonic spasms. Whereas moribund chicks did not manifest any nasal discharge, dead chicks disclosed catarrhal discharges in their sinuses. Upon postmortem examination, considerable amounts of bagasse were found in crops and gizzards. The livers were enlarged about 50 per cent and, when sectioned, appeared markedly congested. Bacteriologic cultures and hemagglutination-inhibition (HI) tests for Newcastle disease (avian pneumoencephalitis) were negative. Unsprayed bagasse, when fed to chicks for a short time, had no adverse effect. When the spray suspension alone was introduced into other chicks, comparable symptoms and lesions were observed.

LITERATURE CITED

According to Schwarte,[1] dry DDT crystals and the water-dispersible preparations, used in reasonable quantities as indicated for their use in insect control, did not cause toxic reactions in domestic poultry. He states, "From the results of experimental studies, it would appear that great quantities of 5 to 10 per cent DDT preparations would have to be ingested, inhaled, or dusted upon the bird to cause an unfavorable reaction." Woodward *et al.*[2] reported 100 per cent mortality due to 0.10 per cent DDT within three to ten days when fed to chicks weighing 57 to 79 Gm. These workers induced total mortality within four to sixteen days when a comparable group of chicks were fed a 0.05 per cent DDT-mash mixture. The symptoms (*i.e.*, nervousness, sensitivity to stimuli, and fine tremors) were observed among our floor-brooded chicks. Rubin *et al.*[3] reported that 0.25 per cent of DDT in an all-mash ration poisoned 100 per cent of adult birds within twelve weeks, and 0.125 per cent was toxic to 90 per cent in the same experiment. DDT also affected adversely the appetite, body weight, egg production, and hatchability. Marsden and Bird[4] fed DDT to 4½-month-old turkeys and observed that as little as 0.075 per cent was toxic when fed during a period of eight weeks. McNeil and Hinshaw[5] did not observe any injurious effect of DDT on poults that had been placed in houses sprayed with 2 and 4 lb. per 100 gal. of water of a wettable product containing 10 per cent DDT in bentonite. Nelson *et al.*[6] reported a detailed histopathologic study of the effects of DDT on several animal species, including chickens.

No papers were located dealing with the toxic effects of chlordan in poultry.

Since symptoms other than those described for DDT poisoning were observed, this investigation was undertaken to observe gross and histopathologic similarities and differences among chicks treated separately with DDT and chlordan. Lethane was also tested, since it was used in the insecticidal spray.

PROCEDURE

The 126, day-old chicks used were "randomized" into seven lots and placed in an electric battery brooder. They were fed a standard chick starter ration. On the seventh day, DDT, chlordan, and lethane were incorporated into this ration and fed continuously for fourteen days (table 1).

These insecticides were dry mixed in proportion to the total weight of the ration. Observations were made every six hours following the introduction of the treated mashes and all dead chicks were autopsied.

Bacteriologic cultures of the visceral organs were made of samples from each group. Com-

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*Dichloro-diphenyl-dichloroethane. Use of DDT, in so far as we know, was first reported on poultry ectoparasites by Tilford, H. S.: DDT Sprays, Country Gentlemen, *116*, (1946):48-49.

**1,2,4,5,6,7,8,8-octochloro-4-7 methano-3a,4,7,7a-tetrahydroindan. Use of chlordan was first reported by Kartman, L., Tanada, Y., Holdaway, F. G., and Alicata, J. E.: Control of Arthropod Carriers of Poultry Parasites, Hawaii Agric. Exper. Sta. Rep., 1946-1948:100-102.

†B B¹ dithiocyano diethyl ether (13.5 per cent by weight and 86.5 per cent inert material). Use of lethane was first reported by Alicata, J. E., Kartman, L., Nishida, T., and Palafox, A. L.: Efficacy of Certain Sprays in Control of Lice and Mites of Chickens, J. Economic Entomol., *40*, (1948):922.

posite HI tests for Newcastle disease were run. The liver and lungs from representative groups were sectioned.

RESULTS

The distribution of death due to the toxicity of DDT and chlordan is shown in table 2. DDT and chlordan proved toxic at both concentrations. In general, both insecticides caused earlier deaths at the 0.5 per cent than at the 0.25 per cent level. As may be seen in table 2, the chicks fed chlordan at either level died earlier and in a shorter period of time than those fed DDT. Lethane did not cause any mortality at either of the levels.

The symptoms induced by chlordan were different from those caused by DDT. The chicks fed chlordan (0.5%) chirped nervously, and those seriously afflicted were down on their hocks or on their sides within twenty-four hours. No apparent symptoms were observed among the DDT-fed chicks to that time, although definite symptoms of toxicity were noted at thirty hours. No nervous tremors or apprehensiveness, particularly to sudden noises, were observed among the chlordan-fed chicks. These chicks, however, did enter terminal agonal stages of excitability. Mucous exudate in the nasal passages was frequently seen in chicks killed by chlordan. DDT, on the other hand, within thirty-six hours, induced marked tremors and hyperexcitability which continued until death. DDT-fed chicks continued to eat until seriously afflicted. Chlordan-fed chicks ate much less feed after they were affected.

Consistent gross pathognomic lesions were not present in chicks that died from either chlordan or DDT. Histopathologic studies of the liver and lungs did not reveal any definite pathologic changes in the 5 birds examined from each group.

TABLE 1—Ration Fed to Chicks

Lot (No.)	Ration
1	Control—no insecticides.
2	Control ration containing 0.50 per cent DDT*.
3	Control ration containing 0.25 per cent DDT*.
4	Control ration containing 0.50 per cent chlordan**.
5	Control ration containing 0.25 per cent chlordan**.
6	Control ration containing 0.25 per cent lethane.
7	Control ration containing 0.125 per cent lethane.

*Genitox, 50 per cent wettable: containing not less than 50 per cent DDT.
**Dowklor, 40 per cent wettable: containing 24 per cent chlordan and 16 per cent related compounds.

TABLE 2—Percentage Distribution of Mortality of Chicks Fed DDT, Chlordan, and Lethane

Hours after add. insect.	Control	Percentage of insecticides in rations: DDT 0.50 (%)	DDT 0.25 (%)	Chlordan 0.50 (%)	Chlordan 0.25 (%)	Lethane (B-72) 0.25 (%)	Lethane (B-72) 0.125 (%)
18	....	....	....	....	....	....	....
24	....	....	....	22.2	....	....	....
30	....	....	....	11.1	16.5	....	....
36	....	5.6	....	11.1	....	....	....
42	....	5.6	....	5.6	27.8	....	....
48	....	5.6	....	5.6	22.2	....	....
54	....	5.6	5.6	5.6	5.6	....	....
60	....	22.2	11.1	11.1	....	....	....
66	....	5.6	5.6	5.6	22.2	....	....
72	....	16.5	11.1	11.1	....	....	....
78	....	....	11.1	11.1	....	....	....
84	....	11.1	16.5	....	....	....	....
90	....	5.6	5.6	....	....	....	....
96	....	....	....	....	5.6	....	....
102	....	11.1	5.6	....	....	....	....
108	....	....	....	....	....	....	....
114	....	5.6	11.1	....	....	....	....
120	....	....	....	....	....	....	....
126	....	....	....	....	....	....	....
132	....	....	....	....	....	....	....
138	....	....	11.1	....	....	....	....
144	....	....	....	....	....	....	....
150	....	....	5.6	....	....	....	....
156	....	....	....	....	....	....	....
162	....	....	5.6	....	....	....	....
% Mortality	0.0	100.0	100.0	100.0	100.0	0.0	0.0

DISCUSSION

There seem to be no symptoms characteristic of chlordan or DDT poisoning of chicks. The discharge in the nares following the agonal stages of chlordan poisoning could be misconstrued as some respiratory disturbance. The nervous manifestations of DDT poisoning may be confused with the nervous forms of other diseases. Together, these insecticides offer many difficulties in the diagnosis of samples submitted at a pathologic laboratory. It is doubtful if a correct diagnosis by gross or microscopic examination could be made on poisonings by these two products without an adequate history.

Because chlordan and DDT are extremely toxic to chicks, it is apparent that caution must be exercised in their use. Strong concentrations should be avoided, especially in areas accessible to chicks. It has been observed that chicks will readily ingest freshly prepared bagasse and therefore the spraying of such a litter with chlordan or DDT should be avoided.

SUMMARY

DDT, chlordan, and lethane were fed to week-old chicks. Whereas, lethane was not toxic at the levels fed, DDT and chlordan were toxic. Chicks fed chlordan died earlier and in a shorter span than those fed DDT.

It was not possible in this investigation to differentiate, either by autopsy or histopathologic studies, between DDT and chlordan poisoning. There were no definite pathologic changes ascribable to either insecticide. It is not likely that a correct diagnosis of poisoning by either DDT or chlordan can be made by gross or microscopic pathology without an adequate history.

This study was not designed to determine the efficiencies of various sprays; therefore, we cannot make any recommendations as to the effective limit that the spray may be used and yet avoid toxicity. Dr. J. E. Alicata, University of Hawaii, is at present working on that problem.

References

[1]Marsden, S. J., and Bird, H. R.: Effects of DDT on Growing Turkeys. Poult. Sci., *25*, (1947): 3-6.

[2]McNeil, E., and Hinshaw, W. R.: Experience with DDT on a Turkey Ranch. Vet. Med., *42*, (1947): 181.

[3]Nelson, A. A., Draize, J. H., Woodward, G., Fitzhugh, O. G., Smith, R. B., and Calvery, H. O.: Histopathological Changes Following Administration of DDT to Several Species of Animals. Pub. Health Rep., *59*, (1944): 1009-1020.

[4]Rubin, M., Bird, H. R., Green, N., and Carter, R. H.: Toxicity of DDT to Laying Hens. Poult. Sci., *26*, (1947): 410-413.

[5]Schwarte, L. H.: Diseases of Poultry by Biester, H. E., and Schwarte, L. H., Iowa State College Press, Ames, 2nd ed., (1948): 1001-1002.

[6]Woodward, G., Nelson, A. A., and Calvery, H. O.: Acute and Subacute Toxicity of DDT (2,2-bis (p-chlorophenyl)-1,1,1-Trichloroethane) to Laboratory Animals. J. Pharmacol. and Exptl. Therap., *82*, (1944): 152-158.

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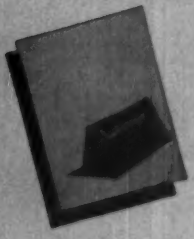

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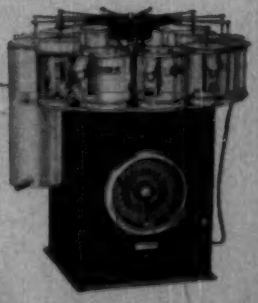

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